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Don't squander precious time on genes

THE House of Commons Select Committee on Science and Technology is one of Britain's more valuable Parliamentary institutions. In the past it has held a number of important and useful inquiries—into Lord Rothschild's proposals for reorganising research council funding, for example, and Britain's choice of nuclear reactors. Even if the conclusions of such inquiries have been open to criticism, they have often thrown much-needed light on some of the murkier corners of government decision-making.

It is, however, difficult to be entirely enthusiastic about the select committee's decision last week to establish a sub-committee on genetic engineering—which presumably means the implications of current research using recombinant DNA techniques. Not that the subject lacks widespread public concern (perhaps more so in the United States than in Britain), nor that it is one on which politicians, in whose lap some of the important decisions may ultimately fall, are well informed. But the timing is wrong.

It is now over three years since the moratorium on recombinant DNA was announced by a group of scientists in the US, and in that time much has happened. Every country which carries out such experiments has now developed some form of control mechanism (some, admittedly, more effective than others). And in the process, the issues

raised by the experiments have been subject to exhaustive scrutiny and debate. As a result, there now seems to be a general feeling that initial reactions may have been too strong. This cooling-off has undoubtedly been partly due to the successful lobbying of those with scientific and other interests in the continuation of such experiments. But there is also a general feeling among many scientists that the dangers, although real and potentially extremely hazardous, have been overplayed.

In these circumstances, it is difficult to see what the select committee will be able to achieve. The area is not one which, like some previous issues, can claim to have suffered from lack of public debate. This is not to claim any particular immunity for decision-making processes within the scientific community. But although such an inquiry would have been useful two years ago, there seem to be more pressing and potentially significant issues at the present time. Examples might be the future needs for scientific manpower, or the definition and management of technological risk in general (which might, indeed, still be embraced by expanding the terms of reference of the new subcommittee). The select committee's time is a precious commodity; it would be a pity if it was squandered on re-opening the scars of previous battles. □

China's path ahead

We bring together in this issue of *Nature* a number of reports on recent developments in science within the People's Republic of China, together with a note on the Peking/Taipei conflict, which is at present reverberating within the international unions. Some of the barriers between the People's Republic and the rest of the world were lifted five years ago. Yet it is still possible for the Chinese to spring surprises on us—with their great wealth of data, their respect for the role of the amateur in some scientific fields, and most recently with the obvious signs of a profound interest in re-establishing science and technology as a thoroughly professional pursuit. This latter trend can be seen not only in the way that university and institutional research is being revived for the purpose of obtaining economic benefits for the nation, but also in the way that certain selected branches of science with less obvious practical benefits are to be stimulated. The all-China meeting in the Spring to discuss national policies on science and technology is bound to be a fascinating occasion.

The path ahead for China is by no means simple, however. Renewing a respect for pure research, for theoretical

understanding as well as practical achievement, even for an elitist rather than populist approach, will not be easy after half a generation in which many of the invisible threads of tradition in the scientific method may have been damaged. But perhaps an even more difficult task will be for the Chinese to hold fast to those elements of people's science which have proved so successful in recent years, most notably the accurate reporting of natural phenomena whether they be connected with health, agriculture or stirrings within the earth. For in some instances this mobilisation of the masses has had major successes—the use of amateur scientists in earthquake prediction, for instance, with many quakes now successfully predicted, puts the Chinese well ahead of the rest of the world.

How maintain a broad public involvement in science and technology while giving the experts their head? And how cope with an almost inevitable demand from a newly-arisen intelligentsia for a greater extension of human rights and liberties? These are among the questions that China must confront in the next decade with its new mood of enthusiasm for science. □



Science in Hua's China

The key to modernisation

Science is undergoing a renaissance in post-Mao China. The United States National Academy of Sciences has been keeping track of the changes, as **Mary Brown Bullock** reports

IN recent months many visitors to China, and the Chinese press, have been recording a dramatic change in China's science and technology policy. Since the "smashing of the gang of four", China has embarked on a pragmatic drive to achieve the "four modernisations"—industry, agriculture, national defence, and science and technology—by the year 2000. The significant aspect of the current use of this slogan is the new emphasis being placed on science and technology as, in Chinese parlance, the "key link" in supporting the other three sectors.

The scientific renewal has been explicitly endorsed in speeches by Chairman Hua Kuo-feng and Vice-Premier Teng Hsiao-p'ing. It has also been evident in a spate of Chinese newspaper articles, scientific conferences and meetings, with the promise of more to come. As a result the broad outline of China's revitalised science policy have become evident.

The new focus includes basic re-

search, selective learning from foreign sources, specialised graduate training, and a new respect for professionalism. In the terms of China's 25-year-old science policy debate, the tension between "reds" and "experts" has eased. Both the masses and the professionals have contributions to make, but the cutting edge has returned to the experts.

Some of these trends were observed by a high-level delegation of American scientists led by Dr Philip Handler, President of the National Academy of Sciences, and sponsored by the Committee on Scholarly Communication with the People's Republic of China, which visited China in June. During visits to five universities and more than 25 research institutes in Peking, Tsingtao, Wuhan, Shanghai, and Nanking, delegation members observed new vitality in research institutes, planning for more specialised graduate education, and a greatly strengthened role for the Chinese Academy of Sciences.



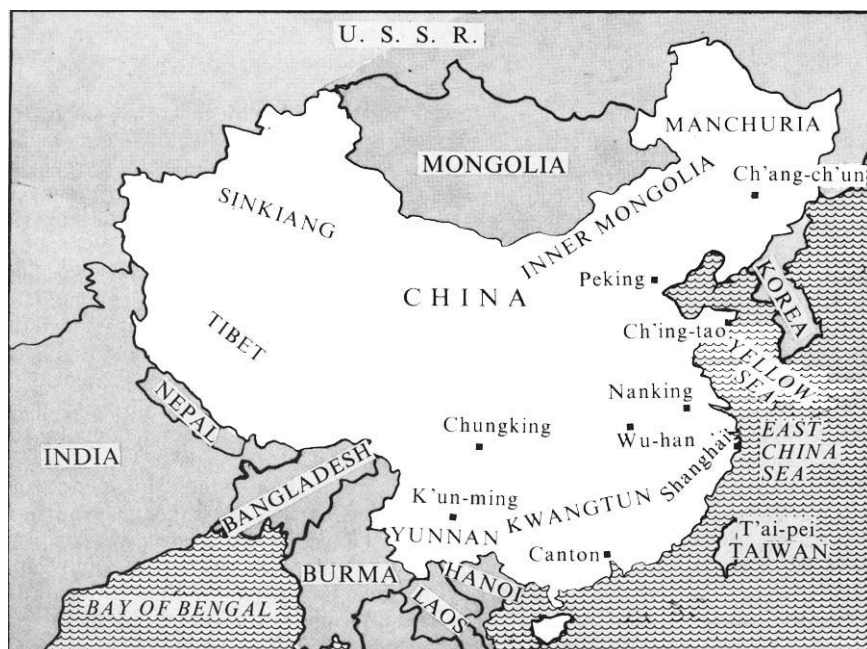
These early impressionistic observations have been confirmed during the past four months by the political reinstatement of China's patron of science and technology, Teng Hsiao-p'ing, by official policies promulgated by the 11th Party Congress, and by numerous articles in the Chinese press. Furthermore, the selection as a member of the Politburo of Fang I, Vice-President of the Chinese Academy of Sciences, has continued to enhance the position of the Academy.

A ministry-ranked institution, the Academy is now being assigned a major role in providing the scientific and technical expertise necessary to modernise agriculture, industry, and national defence. The Academy's research institutes, of which there are over 60, and which were formerly under attack for their 'elitism', now receive encouragement and support from central and local authorities.

New policies

Two articles by leading Chinese scientists published in the authoritative, and usually political, *Red Flag*, reflect a number of the new policies. The first, entitled 'Science and technology must catch up with and surpass advanced world levels before the end of the century', was written by Chien Hsueh-sen, a former aerodynamics and rocket expert at California Institute of Technology, who returned to China in 1955. Published in early July as one of the first detailed discussions of China's new science policy, the emergence of the western-trained Chien as official spokesman itself symbolised a major change.

In a rather candid portrayal, Chien acknowledged a disparity between the state of Chinese and world science. But he went on to affirm that China could catch up through selective, critical



Mary Brown Bullock is with the Committee on Scholarly Communication with the People's Republic of China of the National Academy of Sciences, Washington, USA.

learning from foreign sources, provided that "the role of the professional contingent in the field of science and technology" is brought into full play. He lists four basic areas for improvement: data compilation, scientific instruments, quantification and standardisation, and scientific and technical publications.

The title of the second article, 'Bear in mind Chairman Mao's concern for and teaching on education in science and technology', is almost self-explanatory. Written by Chou P'ei-yuan, vice-chairman of the Chinese Scientific and Technical Association, it cites Mao Tse-tung repeatedly in affirming the importance of theoretical research in the natural sciences and the need to up-grade China's major universities. Chou joins a host of other authors in invoking not only Mao, but more convincingly former Premier Chou En-lai, in legitimising this renewed emphasis upon science and technology.

Science conference planned

Political support for Chinese science is also provided by China's present-day leadership. Chairman Hua has personally addressed a number of scientific conferences, and has called for a national conference on science and technology to be held next spring. But it is Vice-Premier Teng Hsiao-p'ing's imprimatur which is most evident.

One of the key signals that a new policy was in effect, and that Teng was about to be rehabilitated, was the official praising of Teng's 1975 'Outline report on the work of the Academy of Sciences'. Repudiated when written as a "poisonous weed" by the 'gang of four', this article urged raising research standards within the Academy's research institutes.

Not only are Teng's pragmatic concepts now flourishing, but he also appears to be following Chou En-lai's footsteps by taking a personal interest in meeting visiting American scientists, accompanied by senior Chinese scientists. These meetings, announced in the Chinese press, play an important part in enhancing the role of China's intellectuals.

It is too early to identify specific new directions in Chinese scientific and technological research. The conferences which have already been convened at both the national and provincial levels have been on a range of topics, including high energy physics, agricultural sciences, and geology. Seminars have been held on developments abroad in electronics, laser technology, and communications, fields which have featured prominently in international exchanges. As more details unfold concerning a proposed 1978 National Conference on Science and Technology, and as publications ex-

pand, additional research priorities should become evident.

It is also too early to predict how modifying China's former autarky will affect its international scientific and technical relations. It is important to realise that modest exchange between Chinese and Western scientists has been underway throughout the 1970s. For example, over thirty Chinese scientific delegations have visited the United States since 1972 under the centralised auspices of the Committee on Scholarly Communication with the People's Republic of China. Many more scientists and engineers have participated in commercially-related programmes.

Other nations have had similar experiences, and also have begun longer-term individual visits. Significant signs of a changing Chinese posture might be expected to include an increase in Chinese scholars studying abroad, the inauguration of cooperative scientific research programmes, and expanded purchases of foreign technology.

There has recently been an expansion in the numbers of scientists and engineers from all countries visiting China, and they describe a scientific renaissance. Individual Chinese scientists describe themselves as being "jubilant" over the new policies. Hosts have been eager to arrange substantive itineraries and to provide opportunities for foreign scientists to meet individually or in small discussion groups with Chinese colleagues.

During October, for example, a series of joint seminars on specific topics in cancer and astronomy are being arranged for two CSCPRC delegations. Americans hosting visiting Chinese scientists and engineers have likewise remarked on the relaxed and

candid attitude of their visitors. A new dialogue appears underway.

It is perhaps significant that leading Chinese scientists, Chou P'ei-yuan and Pei Shih-chang, were seated at the head table with US Secretary Cyrus Vance and the Chinese Foreign Minister, Huang Hua, during Vance's August trip to Peking. However, since the absence of diplomatic relations may provide some restrictions to programmes with the United States, China's interchange with Britain, Western Europe, Japan, and Australia will probably provide the first signs of major policy change. In this connection, an exchange of high level groups between the Australians and the Chinese Academy of Sciences has recently been planning seriously for longer-term programmes.

Since the beginning of this decade, the developing and industrial world has been fascinated with China's attempts to fashion a science and technology policy which includes the masses, traditional science, and indigenous technology. One need only remember the eruption of interest in the early 1970s when the rest of the world 'discovered' acupuncture and barefoot doctors.

World attention is now focusing on the more technical, professional aspects of China's new policies. Major changes in science and technology policy are certainly underway. But this new emphasis will not entirely replace the heritage of the Cultural Revolution. Chairman Mao's dictum that science should "serve the people" has been refined by Chairman Hua's "May science and technology flourish, and may the good news pour in". The latter part of that phrase, "good news pour in", however, suggests that results, now as before, are still expected. □



Chinese seismologist briefs American delegation on Hsinfeng dam, near Canton

Mass surveys to detect cancer

Eleanor Lawrence, who talked to an American scientist recently returned from China, reports on the particular interest China holds for the cancer epidemiologist

A GROUP of cancer specialists was among the first medical teams from the United States to visit China in the more relaxed atmosphere following the fall from power of 'the gang of four'. And according to one of the team, Dr Robert W. Miller, Chief of the Clinical Epidemiology Branch of the US National Cancer Institute, China turned out to be an aetiological wonderland for the cancer epidemiologist. In particular, the causes of several cancers, unusually common in certain regions of China are becoming amenable to study. The clues come from parallel incidences of similar cancers in domestic animals in the epidemic areas. The parallels have come to light over the past decade as a result of mass surveys. The patterns of incidence strongly suggest environmental causes for these cancers and this is the aspect the Chinese appear to be tackling most vigorously.

Mass surveys and screening, largely carried out by the barefoot doctors and local people, are the most important and most emphasised aspect of China's fight against cancer. The emphasis is on identifying high risk areas, early diagnosis and treatment, and prevention by health education. Where early diagnosis coupled with simple surgery is likely to be successful, as for oesophageal cancer, it is widely practised in the country hospitals and the success rate is high. Radiotherapy and sophisticated chemotherapy is available in hospitals in the large cities but Dr Wu Huanhsing, head of the Oncological Hospital of the Chinese Academy of Sciences in Peking believes that "you can't treat cancer with a big machine".

Dr Li Ping, Deputy Director of the Cancer Research Institute in Peking, showed the American team the first results of a massive survey of cancer mortality, which eventually will cover all of China. Deaths from the commoner types of cancer are recorded commune by commune (each commune consists of about 8,000-10,000 people) and maps made of the relative incidence of each type of cancer within a province. Of the 29 provinces in China, one has already been completely mapped and six more are in preparation. Already sharply-defined areas of high incidence of liver cancer, for

example in southern China, can be seen, whereas lung cancer deaths are distributed non-randomly (as in similar maps prepared in the United States by the National Cancer Institute). Writing in the English language magazine *China Reconstructs* (October 1977) Dr Li estimates that a million barefoot doctors are engaged in the survey.

Mapping high incidence areas

The Chinese have set up some 30 bases for cancer study, prevention and treatment in villages, factories and mining areas throughout China, in areas with high incidences of cancer. The clearest picture so far of cause and effect has been obtained for oesophageal cancer, which has an unusually high incidence in some regions of northern China, focused on Linhsien county. Among the 700,000 people in the county one new case of oesophageal cancer is diagnosed every 8 hours, some 50 times the rate for US whites.

The high incidence of human oesophageal cancer is paralleled by a similar excess of gullet cancer in domestic chickens in the area, leading to the suspicion that both cancers might have a similar environmental cause. Important clues to the possible cause have been uncovered by the Chinese following the relocation of more than 50,000 people from Linhsien county to Changshun county, 350 miles away, where the incidence of oesophageal cancer is low. When the people moved, in 1967-68, to allow a new reservoir to be built, they apparently did not take any of their

chickens with them but bought new birds when they arrived in Chungshan, from flocks hitherto free of gullet cancer. Since then, however, 12 chickens of the 5,500 owned by the immigrants have developed gullet cancer although no cases have been reported in about 2,400 chickens belonging to the native population. This pointed to a carcinogen limited to the chicken's immediate environment, but not apparently confined to Linhsien county. One suspect may be a pickled vegetable mix, a speciality of Linhsien, which could also be fed to the chickens in table scraps. Nitrosamines have been found in this mix and the American team has brought back samples, which will be tested in the Ames test amongst others, for mutagens which may also be carcinogenic.

The American team commented that changing the chicken food from table scraps to grain would provide an unambiguous answer to whether the carcinogen was in food eaten by both chickens and people. The Chinese are indeed mounting a campaign in Linhsien county itself which is aimed at: preventing food and grain from moulding (toxins produced by some mould contaminants are suspected carcinogens), correcting a molybdenum deficiency in the soil, removing excess nitrates and nitrites from water and "changing undesirable dietary habits".

presumably to eliminate food containing nitrosamines. They are also now able to detect oesophageal cancer in its earliest stages by mass screening and the cure rate, by surgery, is apparently very good.

Pinpointing several causes

According to Dr Miller the Chinese are not so interested in pinpointing a specific cause, as in preventing cancer by controlling a range of possible causes. They believe that there is no one specific cause for any type of cancer and that at least four factors will be involved. This perhaps accounts in part for the resistance the American team found to the idea that lung cancer is overwhelmingly caused by cigarette smoking. A substantial proportion of the population in China now smokes and the lung cancer rate is also increasing, although because cigarette smoking only became widespread in China some 20 years ago the full impact has not yet been felt.

Another cancer which has an unusual incidence in China and which also seems to be associated with a similar cancer in domestic animals is nasopharyngeal carcinoma, which seems to cluster in southern China. In a rural area 100 miles from Canton, the American team talked to a barefoot doctor who told them that six people



Storage jars for clean, uncontaminated grain.

This visit was sponsored by the National Academy of Sciences' Committee on Scholarly Communication with the People's Republic of China.

in the 3,000 under his care had developed the tumour in the past decade. Only when they returned to Canton did the American scientists find that nasopharyngeal cancer had been found to cluster in pigs elsewhere in Kwangtung Province—the 'snuffling pig' syndrome. As with the chickens, this could point to a carcinogen limited to the immediate environment of the pig, a much more restricted environment than that of humans and easier to

analyse.

Hepatocellular cancer (liver cancer) is 'epidemic' throughout southern China and the Chinese are looking at hepatocellular cancer and cirrhosis in domestic ducks, although no systematic survey has apparently been made. Studies on the inherited susceptibility to various types of cancer are not apparently given great prominence in China, prevention and early detection and treatment being stressed. But the

American team obtained anecdotal evidence of several instances of very strong familial clustering possibly indicating genetic susceptibility.

The American team also included pathologists, pharmacologists and clinicians and a full report of the visit will be published next year by the National Academy of Sciences. In the present climate of a free exchange of information, it should be one of the largest reports yet. □

Two major controversies

T. B. Tang traces recent changes in China's education policy and the status of her scientists

FOLLOWING the death of Chairman Mao and before the 'gang of four' fell from power, the two major controversies in China concerned cultural activities and the educational system. It was in these spheres that the 'gang' exerted influence for the longest period of time, and it is therefore not surprising that these fields are now seeing the largest and most definite changes, with important implications for the development of science and technology.

One early indication of this was the widely publicised memorial meeting held for Chou Jung-hsin in August. Chou, who died last year, was a former Minister of Education, and had previously held a leading post in the Academia Sinica; it was disclosed that he had been "framed" by the 'gang', and had suffered considerably as a result. An article by the Ministry of Education in the current issue of *Red Flag* now asserts that the educational policies in force during the 17 years before the Cultural Revolution were basically correct. It implies that the "Sixty-Point Document on Higher Education" prepared by the Ministry in 1961, could still be relevant today.

Recent events indicate increased concern over higher education in science and technology. National conferences were held in September on compiling university text-books and, more crucially, on university enrolment. A new periodical, *People's Education*, also started publication in October to serve as a forum for discussing changes in education policy.

One of those changes affects applicants for university places. They are now to take the initiative in putting forward their own names for consideration rather than waiting for selection. Although the universities will continue the "open door" policy of enrolling workers, peasants, demobilised soldiers, and ex-students settled in the countryside, a policy of "walking on two legs" is to be followed, so that direct entry from middle schools is now allowed, and is in fact scheduled to account for about 30% of available places.

In the universities, administrative responsibilities will be delegated by Party branch committees to principals and faculty and department heads, posts which had previously been abolished, but now are being re-introduced ('revolutionary committees')

will be phased out). Entrance examinations which "are necessary in evaluating the political and general educational level of entrants", will also return, being held annually in July or August.

Political education, however, will not be played down; most university students, for example, are likely to spend one month every year living and working with workers or peasants. China continues to encourage expansion of the 'July 21' workers colleges run by factories and mines, and agricultural institutes modelled on the 'Chao Yang' college. They accept peasant-students selected by communes who return there immediately after graduation.

Enrolling research students

It is repeatedly stressed that "to achieve big progress in science and technology it is imperative to arouse the masses boldly"; but, for science at least, more importance appears now to be attached to "the backbone role of professional scientists". The teaching curricula of many universities are being revised, and much effort is being spent on bringing textbooks up to date.

Universities are apparently intended to serve as centres of both higher education and research. Together with institutes under the Academia Sinica, they are preparing to enroll research students for the first time in ten years. Applications will be received this year until the end of December, and post-graduate training is planned to last three years. 1977 graduates will be allowed to take a proportion of the allocated places.

Young people in China are increasingly urged to devote themselves to science. In the rationalist tradition of Marxism, socialist man is substantially equated with scientific man; an additional persuasion now is the call of the four "modernisations", namely agriculture, industry, defence and, most importantly, science and technology.

In November a science week in Shanghai was attended by over two



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T. B. Tang is at St John's College, Cambridge.

million school students. And many leading scientists participated in a meeting of the Chinese Scientific and Technical Association in Peking in August, which was attended by over seven thousand middle school students and teachers of mathematics, physics, and chemistry. Delegates were exhorted to "acquire knowledge to scale new heights in science and our socialist motherland". In a typically Chinese manner, the biologist and popular science writer Kao Shih-Chi composed a poem for the students entitled "Make Science and Technology Serve Our Motherland".

The latest drives in China's educational front are no doubt directed towards economic ends; but they also have political goals. The persistent incentive is not materialistic—though such an aspect exists—but ideological, relying on popular enthusiasm.

This brings us to the position of scientists. The attitude towards professionals and scholars has varied widely in recent times. Up to 1956, intellectuals were allowed to retain the status and privileges traditionally reserved for scholars in China as long as they modified their views to fit the current political mood. From 1960 up to the Cultural Revolution certain Party leaders conceded that academics and specialists were useful whatever their personal ambitions or political consciousness.

After the Cultural Revolution and the advent of the 'gang of four' the policy changed. Intellectuals were considered to be entirely dispensable unless they were "red", which meant usually that they had not been "contaminated" with "bourgeois" ideas abroad, that they had not shown "individualism" by producing scholarly works, and that they were not in prominent positions and earning high wages.

Working hard for socialism

Today, according to the *Political Report*, "the overwhelming majority" of intellectuals "are willing to work

hard for socialism and are indeed doing so". Political apathy will not be allowed, and help and encouragement will continue to be given to intellectuals "to remould their world outlook"; at the same time, however, they will be guaranteed the conditions to put their abilities to good use.

The "Circular on the holding of an All-Nation Conference on Science", issued in September, announced the restoration of titles for technical personnel as a means of enhancing their sense of responsibility. The Academia Sinica, for example, now has research assistants, and assistant, associate, and full research fellows. An increase in wages took place at the beginning of October, with scientists—among others—given priority for individual recommendation.

The *Political Report* states that one of the aims of the forthcoming All-Nation Conference is to "commend the pace-setters, especially those scientists and technicians as well as workers, peasants and soldiers who have inventions and innovations to their credit". The practice of awarding orders and decorations to scientists, first introduced in 1956, may also be restored.

The *Political Report* also suggests that the Chinese people should unite with "our countrymen overseas"; it appears that a State Department for Overseas Chinese Affairs may soon be formally set up. The main contribution of ex-patriate Chinese, of which there are about 20 million, will be to act as bridges of friendship; but in particular cases they can do more.

This year, for example, has already seen the visit of C. S. Wu and the three American-Chinese Nobel prizewinners in physics. For Yang Chen-ning it was his seventh trip, and in the last few visits he has collaborated on important research projects. Another collaborative research project by Chinese scientists at home and overseas is the research on the heredity role of cytoplasmic mRNA, in which M. C. Liu and P. Y. Pang from Temple

University took part.

International collaboration

Perhaps significantly, the *People's Daily* published on 3 October a poem entitled "A returned scientist". But whether any overseas intellectual wishing to return to China, either short-term, long-term, or permanently, is able to do so remains essentially a political decision.

The international transfer of science and technology takes many forms. The most obvious ones are importing instruments, machinery, and complete plants, and acquiring information through the circulation of journals and reports, and through private communication. But this transfer also takes the form of international movements by scientists and engineers.

Many in the West will have, through direct or indirect contact, felt the expanding number of exchange visits with China. In addition, we have been given to understand that, at least for Chungshan University (situated in Canton and one of the 28 "key universities"), visiting professors may soon be welcomed, particularly from the Second World. And here the consideration will be mainly academic.

Chungshan University has announced that it is arranging joint research projects with the Polytechnic of Hong Kong. This collaboration may be viewed primarily as a social relations exercise, but it is an important step.

Another development, reflecting the emergent liveliness in Chinese scientific circles, is the recent appearance in *Acta Astr. Sinica* of a paper contending that the universe is closed. Apparently contradicting the Marxist dictum that the universe is not only boundless but also infinite, the paper might well have been refused publication, a few years ago.

Recently, there have been many changes in China's science and education policy. Most—if not all—are not reversals of previous ideas, but a renewed emphasis on policies formulated in the era of Chairman Mao. The changes are necessary not only to repudiate the manoeuvres of a group now fallen from power, but also to prepare China for the conditions she is likely to face in the near future.

In developing her science and technology, China as a Third World country has had 28 years of useful experience which constitutes a basis on which she can fruitfully draw for future programmes. She is endeavouring to develop a modern technological society modelled neither on Western countries nor on Russia and is taking a path never previously followed. In China as in the West, however, there are many problems ahead, not the least being the social impact of progress in science and technology. □



Looking to the stars

Professor Graham Smith, the British astronomer, visited China this autumn and returned feeling China needs a large injection of Western expertise

ANY scientist must be encouraged to hear that his government is in favour of a rapid expansion of his subject, and that adequate resources will become available. It must, however, be a daunting prospect when excellence is required within a few years, especially when a lack of contact with contemporary science, and a long history of neglect and social turmoil, provide a starting point far behind the world's front runners.

The training of astronomers in China has suffered in recent years alongside all areas of university education. Nevertheless astronomy, and especially radio astronomy, is a well favoured subject in contemporary Chinese science. There is both a determination to achieve excellence in at least a handful of research fields, and an unprecedented encouragement of contacts with the West.

On my visit to China in October as representative of the Royal Greenwich Observatory, I was accompanied by Professor Wynne, who was particularly interested in optical design. We therefore not only visited the major observatories but also the major optical institutes and factories, in most of which Wynne's work on computer optimisation of lens design was being adopted.

The main observatories in China are at Shanghai, Nanking, Peking and Kunming. Shanghai Observatory is mainly concerned with measuring time through the use of transit telescopes and astrolabes. It has an astrolabe of novel and advanced design, recently described in *Acta Sinica Astronomica* (an English translation of this journal is now available). Although Shanghai is a centre for the time service in China, it has no caesium clocks, and China takes no part in international efforts to maintain the Atomic Time Scale.

Nanking has the famous Purple Mountain Observatory, where some of the most spectacular of the fifteenth-century bronze instruments are preserved, and some astrophysical work and satellite tracking observations are now carried out. Peking Observatory has several departments, notably the optical observatory at Hsin Lun, 960 m above sea level and 110 km NW of Peking, where there are several working telescopes, including a 60 cm Cassegrain recently built at the Astronomical Optical Factory of Nanking. The same factory is now undertaking the construction of a 216 cm telescope

for Hsin Lun.

The radio observatory of Peking is at Mi Yun, about the same distance from Peking. The site is well chosen on a flat plain screened by hills from interference, and has a 1 km array of 9 m reflectors. And at Kunming, in the south-west of China in Yunnan province, there are solar and astrometric telescopes, and an active observatory which recently started a large expansion.

Peking University has no research school in astronomy, even though radio astronomers are taught there. Their training has consisted of a third undergraduate year in radio astronomy, in which practical experience with a very elementary solar radio telescope played an important, but hardly advanced, rôle. The modern techniques of aperture synthesis and very long baseline interferometry (VLBI) were often mentioned, but there is naturally no direct experience of such important ingredients of modern radio astronomy, and no plans have yet been formulated of how to start.

The radio observatory at Mi Yun is the obvious nucleus for any expansion in the subject. The 22 paraboloids of the 1 km array have recently been rebuilt, increasing their diameters from 6 m to 9 m, and are used as a phased array at frequencies of 450 MHz and 176 MHz, observing the sun. The angular resolution is to be increased by the addition of further paraboloids, spaced at further intervals of 1 km, and used in a switched system.

The next development planned would be to rebuild the whole system for use in aperture synthesis, although this would be a very large task, involving amplifiers and separate cables for each paraboloid, a new complex receiver system considerably more sophisticated than the present simple switching receiver, and an on-line computer.

My own view is that rapid progress of the necessary order could only be achieved by a determined infusion of ideas and techniques from the world's large radio observatories. The problem of language is serious, since few visitors could even hope to learn Chinese, and surprisingly few of the Chinese astronomers speak English well. The main problem, however, is the lack of students and young research workers with any familiarity with modern work. It is essential for some of the best of young Chinese astronomers to spend considerable periods abroad; possibly two years in a major observatory would



Fifteenth century astrolabe at Purple Mountain Observatory

be needed.

Although radio astronomy is at present the favoured subject, progress might be better made by concentrating on optical, or possibly on millimetre wave astronomy. The site of the Peking optical observatory is not particularly good, although it may be the best within reasonable distance of Peking. But there must be some very good sites in China, especially at the high altitude which millimetre wave astronomy demands.

Kunming itself is at 2,000 m, and the skies are clear, while not far from the city are mountains over 2,500 m which might provide good sites. There is undoubtedly a wealth of good astronomy to be done with moderate sized optical telescopes; the worrying aspect of radio astronomy is that middle-sized radio telescopes may be no use in comparison with the giant arrays now being used, or constructed, in a few major observatories.

In the observatories and institutes which we visited there are plenty of people, and there are new buildings which at Kunming reach a lavish scale. There must be room for a staff of at least 500 when the buildings are complete even though the observational work in progress at present would occupy a staff of only about 50 at an established observatory. Optical design is becoming well understood in China, and the techniques of optical working now extend to the figuring of mirrors up to 2.5 m in diameter.

Instrumental design has been well demonstrated in the new astrolabe constructed in Nanking and used in Shanghai and Kunming. Spectrograph design needs study, but gratings are now available which are ruled at Chang-Chun, in northern China. Low expansion glass is now made in Shanghai, although only in pieces up

to 1 m in diameter. (The 216 cm mirror is a piece of pyrex made in Russia, and presumably imported almost 20 years ago.)

There is much enthusiasm and a good-will towards astronomers which is refreshing and helps to make a visit to China an extremely pleasant occasion. The Chinese want to make real

advances, and recognise that to do so they must participate in the international exchange of ideas which is an outstanding characteristic of astronomy.

It would be very helpful to China's scientists—and surely very good for international good will—if astronomers were sent abroad for extended periods

of study. The regeneration of Chinese science without such contacts will otherwise be a slow and uncertain process. Whether it occurs in radio or in other branches of astronomy will only emerge later, when there are sufficient trained young astronomers able to make their own educated choice. □

Divide between Peking and Taipei rules out scientific cooperation

The issue of the two Chinas is gradually concerning more scientists as the Peking government seeks membership in the international unions at the expense of Taiwan. Richard Harris, of The Times, who lived in China before and after the Communist government came to power, describes the historical background and explains why a two-China policy is unacceptable to either government.

IN 1895 China was ignominiously defeated in a brief war with Japan over Chinese suzerainty in Korea, and as a result Taiwan had to be ceded. China felt this defeat by Japan much more shameful than either of the earlier Anglo-Chinese wars in the nineteenth century. Moreover, emotions were stronger in 1895 since by then a slow response to western intrusion had evoked a nationalism never before felt in China.

The two decades following that defeat (which included the actual fall of the Ching dynasty in 1911) brought home to the Chinese the need for their twentieth-century revolution, and defined its objectives. These involved the regeneration of China—the Chinese term used for revolution has this meaning—so that it would again be united, independent and strong.

United meant the return to the sovereignty of one government over all territories from which such sovereignty had been lost by China from 1840 onwards. Independent meant the abolition of any privileges otherwise acquired by foreigners—such as the treaty ports and the customs control exercised in accordance with “unequal” treaties. Strong meant a China militarily and industrially the equal (if not the superior) of any country in the advanced western world.

Ever since 1895 the loss of Taiwan has remained indelibly in the Chinese mind as a shame to be expunged. Along with other causes for shame, the emotions of Chinese nationalism survived unappeased in this century until

the first government strong enough to pursue the national objectives emerged in 1949. As the communist armies moved southwards in that year, all patriotic Chinese welcomed the end of foreign privilege, and foresaw that Chiang Kai-shek's retreat to Taiwan could only be temporary since all his support had gone and his troops were demoralised.

Suddenly, however, in quite unforeseen circumstances, hopes of a united and independent China were dashed with the outbreak of warfare in Korea (not of China's making), and President Truman's decision to place American power between Taiwan and the intended assault on it from the mainland.

Unlike the situation with East and West Germany, the incoming revolutionary government of 1949 therefore found itself facing exactly the same circumstances as earlier new dynasties when pockets of support for the defeated dynasty were still resisting. Such redoubts of resistance had always maintained their own legitimacy; by so doing they challenged the legitimacy of the incoming rulers in Peking.

Moreover those who thought they had seen the end of foreign privilege and foreign interference in the affairs of China now saw just such foreign interference reasserted in favour of Chiang Kai-shek. An American security treaty soon assured the island against attack, while American hostility to the People's Republic for 20 years impeded diplomatic recognition, and the occupation of the Chinese seat in the UN by the Peking government, which was retained for the government in Taiwan.

As seen from the west, much has changed since 1950. Taiwan's economy has shared in the growth area dominated by Japan. The circumstances of the Korean War have been forgotten. Why should Taiwan be “handed over” to the communists, it is asked. Furthermore, now that the United States has changed its policy of containment towards China; and has established a

mission in Peking, why should China not agree to American requests that the government in Peking should renounce any intention of occupying Taiwan by force?

No one with any first hand experience of Chinese nationalism, or knowledge of Chinese history, could expect the Chinese to meet such demands. Consider them in reverse: would the Americans, solely at China's request, be prepared to renounce action of some kind about territory that was part of the United States?

Another argument offered by westerners is to use the German analogy, and to ask why Peking should not be content to allow Taiwan a comfortable independence. Those who suggest this, however, do not realise that the government of the Kuomintang Party in Taiwan shares all the attitudes of the government in Peking. Each government claims to be the government of China; once the government in Taiwan renounced that claim, it would lose its *raison d'être* even if its reconquest of the mainland is an absurd ritual declamation.

Whatever the arguments, there is no possibility that action by the west, by the UN, or by any other outsider can determine the future of this question. And of those who cling to the hope that the “Taiwanese”—who certainly do not like the dominance of Kuomintang mainlanders, and who find that dominance has not been much mitigated by the greater association of Taiwanese in government in recent years—will assert their wish for independence, one can only ask whether the record of Chinese history gives them any support at all.

Foreign intruders have ruled parts of China at many times, and all of it in the case of the Mongols and Manchus (Ching). But at no point has the concept of unitary government for all persons who are Chinese ever been questioned—not even by the warloads of the twenties. Supporters of a “two-China theory” have been, are and always will be anathema to the government in Peking.

This should explain why neither government will entertain any contact with the other, and why the battle between them in all international organisations, from the UN downwards, will allow no co-existence. □

Israeli scientists woo Egypt

AMONG the documents that Egyptian President Anwar Sadat took back with him to Cairo after his recent extraordinary visit to Israel was a list of 36 international scientific conferences due to take place in Israel this year. Appended to that list was a request that Sadat facilitate the participation of Egyptian scientists in each of these conferences.

A much more far-reaching proposal for scientific cooperation was made immediately after Sadat's departure by Technion Professor Josef Rom, an aeronautical engineer now serving as a Likud member of the Knesset. Rom said that the current Israeli-Egyptian political dialogue should be accompanied by concrete measures to foster joint programmes in as many spheres as possible, among them science and technology. "For example," Professor Rom declared, "we must begin to plan and if possible to carry out bi-national projects such as the construction of atomic power stations and desalination plants in Sinai".

Such a scheme, in fact, was proposed many years ago by Dwight D. Eisenhower, and endorsed by other American leaders, all of whom thought it could help bring peace to the Middle East. Only later did it become clear that in the absence of peace, co-operative development projects were simply not feasible.

But before that scheme was finally shelved, American, Israeli and Egyptian scientists had come together at the Oak Ridge National Laboratory, then headed by Dr Alvin Weinberg, to work out details of how it could be carried out. They envisaged the installation of

two reactors with a combined output of 1,000 MW in the El Arish area, south of the Gaza Strip. The power generated was to be used by newly established industries producing chemicals, fertilisers, plastics, and aluminium, as well as for the operation of a plant to desalinate 1,000 million gallons per day of seawater—enough to irrigate some 300,000 acres of land in the deserts of Sinai and the neighbouring Negev. It was assumed that this American-financed programme would bring great material benefits to both Israel and Egypt and also provide employment opportunities for Arab refugees in the Gaza Strip.

In the interval most of these refugees have obtained jobs in Israel and many other things have changed. But Dr Gerald Stanhill, an Israeli scientist who helped work out some of the agricultural aspects of the scheme, hopes that it will be revived in one form or another.

At the same time, Dr Stanhill warns against the dangers of prematurely promoting "intensive, California-style agriculture" in a country like Egypt because this would involve an enormous investment in money and trained manpower as well as flooding Egypt's already overcrowded cities with "fellahs" who would lose their jobs in the countryside and fail to find new ones in the cities.

But quite independent of the Sadat visit, Stanhill and his colleagues at the Volcani Centre of Agricultural Research have been considering how low-technology irrigation techniques similar to those used on Israeli moshavim (smallholders' settlements) could be

introduced into the Egyptian countryside in a way that would substantially improve living standards without destroying the existing fabric of society. And these techniques—together with those from dozens of other countries—are being brought to the attention of the world's farmers and researchers through the efforts of the International Irrigation Information Centre (IIIC) located at Volcani.

Even so, personal contact is bound to be a more effective means of communication. This can be seen in the Gaza Strip, controlled by Israel since 1967. There Egyptian-trained agricultural instructors were taught Israeli techniques in irrigation and other spheres, and then brought them to local farmers. As a result, average annual production in agriculture has grown by 25.4% (as compared to 6–7% in Israel), while income per farmer has risen from \$130 to \$732.

There is enormous enthusiasm among Israeli researchers—be they working in agriculture, medicine, electronics or geophysics—to establish close contacts with their counterparts in Egypt, by far the largest and most developed scientific centre in the Arab world.

Weizmann Institute President Michael Sela was undoubtedly speaking for many others when he said this week that the Institute "soon hoped to have visiting scientists not only from Berkeley, London, Paris, and Tokyo, but also from Cairo and Alexandria". "Moreover," Professor Sela added, "if our researchers can spend their sabbaticals on the banks of the Thames or the Seine, there is no reason why they shouldn't be spending them on the banks of the Nile".

Nechemia Myers

Euratom burns its public relations' fingers

RELATIONS between the European Commission and the environmentalist lobby got off to a bad start on the first day of last week's public hearings into nuclear energy with the publication of a report purporting to show that the European Atomic Energy Community (EURATOM) was planning a propaganda campaign to sway public opinion in favour of nuclear energy.

The "evidence" for this claim was a report "Design of a Task Force to Build Public Awareness and Support for Nuclear Power", prepared under a Euratom study contract, which was produced at press conferences held simultaneously in Brussels and Rome by the Italian Radical Party and anti-nuclear lobby.

The report, prepared by Alessandra Ovi of the Nuclear Engineering De-

partment at the Polytechnic Institute of Milan, and written in November 1976, described the details of a communication process which would "successfully tackle" public opposition to nuclear power.

Techniques suggested for achieving this included making use of the "authority and credibility" of senior members of the medical hierarchy to diffuse a pro-nuclear message. The report recommends setting up a task force of experts who would "design adequate messages" to provide a coordinated response to nuclear opponents.

The commission's first response to the Italians' charge of manipulating public opinion was to dismiss the document as a "forgery". Later, however, when copies of the document

were produced bearing the EURATOM Study Contract Number 605-76-03, the commission admitted its authenticity.

But according to the commission, the document bore no relation to official policy. It had apparently been a sub-contract made under a much larger contract (to which the contract number referred) on the general assessment of nuclear risk.

The report had been immediately disowned by EURATOM when its existence had become known in June of this year, with firm instructions that the line of inquiry it indicated should not be pursued further. How it got into the hands of the Italian environmentalists is not known; but its very existence, and the swift reaction which it aroused from the commission, was sufficient to add an initial jarring note to the three-day hearing.

David Dickson

New broom at the DHSS

EFFORTS to streamline the administration of medical research have been promised by the new chief scientist at the UK Department of Health and Social Security, Professor Arthur Buller.

The promise is likely to be widely welcomed by Britain's medical science community. There has been growing frustration in recent months at the burdens imposed on research by the implementation of Lord Rothschild's proposals for research council funding.

Professor Buller brings to the job a growing awareness that the Medical Research Council (MRC) and the DHSS must work closely together, and with mutual trust, if the new system is to be made to work. Only this, for example, will avoid the DHSS presenting the research council with requests—such as a “cure for schizophrenia”—that are impossible to meet at the present time.

A close liaison will also, Professor Buller feels, avoid a repetition of the situation that arose two years ago when the Government's economic problems led to hastily imposed cutbacks of department research budgets, and consequently a reduction of £900,000 in the money available to the MRC from the DHSS.

“It is impossible to maintain any type of meaningful relationship between the two bodies if this type of thing, which happens when administration gets quite separated from research, is liable to take place. From the MRC's point of view, last time was a near disaster.”

Professor Buller, whose appointment was announced last week, will take up his new position with responsibility for a £28m research budget from the beginning of January. He is keen to reduce the extra administration that the Rothschild proposals have involved, a desire he shares with the new secretary of the Medical Research Council, Professor James Gowans.

Unlike many scientists, however, he does not criticise the philosophy behind the proposals, under which about 25% of the MRC's £52m research budget is now allocated by the department to the research council on a “customer-contractor” basis.

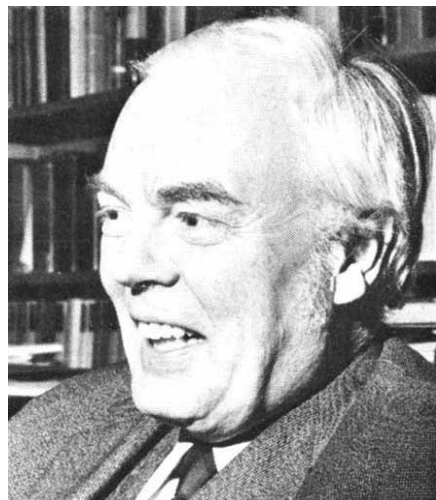
According to Professor Buller, the problem lies in the way that this has been done. “In the past, too much of a meal has been made of being seen to implement Rothschild. An enormous exercise, which has been expensive and time-consuming for both scientists and administrators, has been mounted so that both sides could demonstrate that they were carrying out Rothschild's recommendations”, he says.

By cutting down on this type of activity, while concentrating on what remains necessary to maintain an effective partnership between the MRC and the DHSS, Professor Buller hopes to simplify the administrative system between the two bodies, without any loss of effectiveness. And it is a goal which, he claims, is already shared by the DHSS.

Professor Buller has been seconded to the department from the University of Bristol, at which he is professor of physiology and dean of the faculty of medicine. He shares a general reputation claimed by the university for pragmatism rather than politics in administrative affairs, although membership of the non-academic staff salaries committee has made him no stranger to the latter.

Buller gained further experience in the world of medical politics at the MRC itself, where he is at present chairman of the neurobiology and mental health board and a council member. The board's main concern in recent years can, he says, be succinctly summarised: “We have spent our time trying to live with Rothschild”.

One of Buller's aims likely to receive widespread support among scientists is his desire to return the initiative on research back to the research worker. The most important thing, he stresses, in avoiding the waste of money is to get good scientists doing research, and then



Arthur Buller, new chief scientist

to a certain extent to follow their judgements.

The real need is not the development of ways of directing research from outside, but the successful application of its findings. “We need to keep a very sharp eye on ways in which the research being done by scientists in ivory towers can be exploited for the improvement of health care”, he says.

“In the past a number of important discoveries have lain fallow and not been exploited. Penicillin is an example where a failure to pick up the possibilities early enough meant that the industrial lead was given to the US. I am very keen to avoid this type of thing in the future”.

David Dickson

Indian science reorganised

THE Indian government has now issued a list of the 17 laboratories that it intends to detach from their present position with the Council of Scientific and Industrial Research (CSIR) and relocate within user ministries (10 November, page 89). They are: ten research associations (to Industry), three museums (to Education), the Indian Institute of Petroleum (to Petroleum and Chemicals), the Road Research Institute (to Transport and Shipping), the Central Fuel Research Institute (to Energy) and the Building Research Institute (to Welfare and Housing).

In contrast to the earlier fears that the government would dismember CSIR and shatter morale, first reactions seem to be relief that the transfer has only affected laboratories most easily housed in a ministry. □

Following a Maoist path

Romanian science is being systematically ruined “on the Chinese model”, according to Dr Mihai Dediú, a Romanian mathematician recently exiled with his family to Italy after his cam-

paign for genuine scientific activity.

Although throughout the Comecon block social doctrine states that science must serve the economy, in most states this is no more than a slogan; pure research continues following Brezhnev's dictum that “there is nothing more practical than a good theory”. In Romania however, following President Ceausescu's visit to China, a new trend began by which all institutes of pure research were gradually turned into what Dr Dediú describes as “little more than factories”.

Chemistry was the first to suffer—there is now no faculty of chemistry at all, only chemical engineering. The mathematics institute was destroyed in 1975 and some of the mathematicians were transferred to the Institute of Physics, some to industry. The Institute of Physics, in its turn, became the Institute of Physics and Instrument Production in autumn 1976. According to Dr Dediú, the Academy of Sciences no longer exists as a scientific forum. The whole emphasis is upon politics. Even Madame Elena Ceausescu, the wife of the leader, has been appointed an academician although she has no scientific training.

Dr Dediu himself was involved in these changes, being employed at the Mathematics Institute at the time of its destruction. He was then transferred to the Physics Institute, but was dismissed before it was re-modelled.

Vera Rich

Change into genes

Britain's House of Commons Select Committee on Science and Technology is to set up a subcommittee on genetic engineering under the chairmanship of Mr Arthur Palmer, Labour MP for Bristol North East. The members of the committee will be announced shortly, and it is expected that the subcommittee will begin public hearings—to which Ministers are likely to be invited to give evidence—early in the new year.

Conserving energy

THE European Commission in Brussels is considering plans to set up a group within the energy directorate concerned directly with the problems of energy conservation.

The suggestion for such an initiative was made by various environmentalist groups during the public hearings on nuclear energy held in Brussels last week, the first of a series of such hearings on issues related to energy policy.

At the end of the three-day hearings, the chairman Dr Guido Brunner, who is commissioner for energy, announced that the proposal would be seriously considered by the commission, and that it was likely to be put into effect.

The hearing, which concentrated particularly on future energy requirements, saw a number of confrontations between the pro- and the anti-nuclear lobbies, with many well-worn positions being rehearsed by either side.

Putting the case for nuclear power, for example, Dr Rudolf Guck of Badenwerk AG suggested that the declining supply of fossil fuels, leading to a steady climb in prices, would make nuclear energy an increasingly attractive economic proposition.

According to Dr John Chessire of Sussex University, however, current forecasts of future electricity demand were too high. In addition, whatever the price of uranium, fast breeder reactors would, he claimed, be uneconomic compared with thermal reactors.

Criticism of the European Commission's own energy policy as being "obsolescent" and ignoring "the real needs of society" came from Dr Peter Chapman, director of the Energy Research Group at Britain's Open University.

In a presentation that appeared to make a particular impact on Dr

Prophet of nuclear doom

Mr Tony Benn, UK Secretary of State for Energy, appeared to enjoy last Friday's debate in the House of Commons on nuclear energy.

The debate was brought by Pontypool MP Mr Leo Abse, whose constituents, he said, live within 50 miles of "one of the largest concentrations of nuclear reactors in the world . . . eight reactors in operation . . . and two more planned".

Abse, who had been briefed by Tom Burke of Friends of the Earth, was demanding a government response to the Flowers report, which indicated the dangers of a headlong dash to dependence on nuclear power. Not mincing words, Abse raised the question "is a plutonium based economy compatible with democracy?" and addressed it with some eloquence.

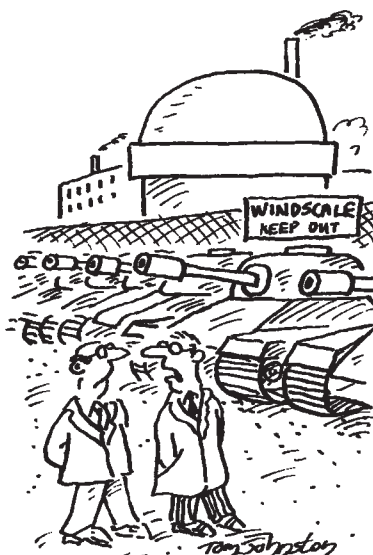
Abse is not exactly pro-nuclear. He

but not against nuclear energy as such. His points were cogent—as indeed are many of those raised by the Friends of the Earth. Tellingly, Benn was delighted with Abse's speech, which he thought "in a classic prophetic mould". Abse's, said Benn, was a "philosophical, penetrating, and perceptive speech, and I hope that it will be widely read and studied". Benn was at pains to point out that in matters of such difficulty it was worth listening to everyone and that "the pressures that are brought to bear are not—as is sometimes suggested—only those brought by the environmental lobby against the innocent nuclear power lobby". Benn went on "In my political life I have never known such a well-organised scientific, industrial, and technical lobby as the nuclear power lobby. It is not so much the Friends of the Earth as what Eisenhower might have called the nuclear industrial complex of which I am aware as a Minister."

With that view, one might have expected Benn to be more forthcoming on the question of an inquiry into the proposed building of a commercial prototype fast breeder reactor in the UK, the £2 billion CFBR1. Mr Peter Shore, Secretary of State for the Environment, has promised a fast breeder inquiry separate from the recent Windscale planning inquiry, which investigated the extension of reprocessing facilities at Windscale. Asked if the government had indeed committed itself to an inquiry on the fast breeder Benn said "We have not yet decided to set up the inquiry". And yet he added "We shall not reach a decision (on the CFBR1) until there has been an inquiry".

Mr Tom King asked for confirmation that the government would not take a decision on fast breeders until the inquiry, and suggested that the Minister had not even discussed the question of an inquiry with other Ministers. Is there progress towards an inquiry, he asked? Benn, apparently cornered, answered "I cannot undertake that there will be a debate . . . when we have settled the thermal reactor question I shall be better able to answer".

Robert Walgate



'UNFORTUNATELY, MOST OF THE PLUTONIUM WE PRODUCE GOES TO POWER OUR SECURITY SYSTEM.'

ended "If we, avaricious for the apparently easy wealth that nuclear energy could bring, enter into a Faustian pact with a meretricious atomic destiny, we may have doomed our children or grandchildren to the loss of their liberties, if not their lives".

But that style is very much Mr Abse's. In more sober moments he took a view broadly against plutonium,

Brunner and other members of the commission present, Dr Chapman claimed that the commission's policy was at least four years behind that of the UK, and that the supply and demand forecasts which it had been putting forward were inappropriate, and of a "very low standard".

"There seems to have been no

analysis of the economics of nuclear systems and no awareness of the importance of storage and transport costs", said Dr Chapman.

The next public hearing, which will be held in Brussels on 25 January, will discuss some of the problems raised by the safety aspects of nuclear energy.

David Dickson

correspondence

What about the peace prize?

SIR,—I am not surprised that, in the normal way, the only Nobel Prizes to which you pay attention (27 October, page 744) are the prizes for physics, chemistry and physiology and medicine. This year, however, scientists have cause to take particular pleasure in the award of the Peace Prize. The 1977 Nobel Prize for peace was awarded to Amnesty International, who—as your own columns have not infrequently reported—have often been involved with helping scientists among the many prisoners of conscience for whom they work.

J. A. EADES

University of Bristol, UK

Limited tenure

SIR,—Both the present Director of the Imperial Cancer Research Fund, Dr Michael Stoker, and his successor, Professor Walter Bodmer, are on record as believers in “the virtues of limited tenure as a means of maintaining high quality in research”. These are fine, high-sounding epithets, but what they actually mean for scientists carrying out research during short contracts is something totally different.

The majority of indigenous talent in cancer research in England is recruited at the doctoral or immediately post-doctoral levels; very often the “virtues of limited tenure” brutally mean—get out by the age of thirty, or certainly not much later. Nobody engaged in activities other than scientific research in the same institute, from the administrators to the catering staff, receive such cavalier treatment; indeed, it would be against the law. Often the PhD student or post-doctorate will feel it necessary to justify himself to his superiors by working long hours, to the detriment of his social life, if not his health. Certainly this will maintain high output, if not also “high quality in research”. In addition, the financial return compares badly with many other professions, quite apart from such incidental mishaps as the refusal of building societies to grant mortgages because an income cannot be guaranteed for the appropriate period of time. And of course the searing intellectual corrosion, the shame, the agonising losses of self-confidence and self-respect that characterise the phase of unemployment and readjustment—inevitable in

many cases—these are the price each individual has to pay for having done a highly skilled job cheaply and to the best of his ability.

It is perhaps unfair to single out the ICRF for blame in a situation in which the market for research scientists is presently glutted by talent of an exceptionally high order in virtually all scientific disciplines; in which the governing principles of employment in grant-aided research for those not medically qualified are primarily cheapness and expendability as opposed to merit or achievement. It is easy to spread an ambivalent gloss over the realities of the situation by glamorising the advantages of short-term appointments as a means of achieving academic excellence, though even this is a highly questionable thesis. Conversely, it is perniciously wrong that gifted young people, often guided into cancer research for reasons that have nothing in common with self-advancement, should be called upon to pay such a bitter and a heavy price for their altruism.

ROBERT JONES

Tübingen, West Germany

Funding of basic research in chemical thermodynamics

SIR,—The membership of the 1977 Calorimetry Conference is deeply concerned with the continuing decrease in funding of research in chemical thermodynamics, especially thermochemistry and thermophysics, and the resultant decrease in data productivity in this field in the past several years. Examination of publications and of Calorimetry Conference programmes shows the greatest decrease to be in the areas of heat capacity measurements and of heat effects in chemical reactions (combustion calorimetry, solution calorimetry, etc.). These are the primary sources of new thermodynamic data for science and technology.

In North American universities, experimental expertise is in danger of being lost in important areas, particularly combustion calorimetry. Programmes in heat capacity calorimetry and in reaction-solution calorimetry are also suffering. Attention is called to the following points:

The production of fundamental thermo-physical and thermochemical data for new materials, and for materials not previously

studied, is not now sufficient for the evaluation of new processes and applications. Many key areas are no longer being studied. These fundamental data are required for effective research and development in virtually all aspects of energy and technological materials programmes.

The precise determination of the appropriate properties of carefully selected ‘key compounds’ is important because the data provide anchor points for making critically evaluated estimates and correlations of the properties of whole classes of compounds. This allows the data base to be expanded greatly on the basis of a relatively small number of carefully chosen measurements.

Acquisition of new data of technological significance on a realistic time scale requires continuous funding to keep available the highly sophisticated equipment and manpower in laboratories necessary for energy-related measurements, in other words, to maintain capability. The initial investment that has already been made could be lost if sufficient funding is not continued.

Continued acquisition of fundamental calorimetric data has been demonstrated as an important endeavour in the solution of practical problems in many areas of science and technology. It is an economical endeavour, leading to increased efficiency in the choice of materials, the design of production facilities, energy conservation, and the selection of appropriate large-scale technology.

Fundamental thermodynamic data are also necessary for the critical testing and evaluation of theoretical advances in chemistry.

The conference urges that governmental, private, industrial, and university laboratories, in the light of the above comments, consider their anticipated needs for chemical thermodynamic information and identify the level of support required (manpower and facilities) to supply the information at a rate commensurate with the need.

Particularly where substantial funding reduction has occurred—perhaps originally as a temporary measure—consideration should be given to restoring support on a continuing basis.

Assurance of multi-year support rather than a strict year-by-year support is recommended because of the resulting economies and because the production of high quality data requires a continuity of trained and experienced experimentalists.

Yours faithfully,

CHARLES E. HOLLEY

Calorimetry Conference Committee,
University of California, USA

news and views

Ribosomal gene organisation in *Tetrahymena*

from Carol Klukas

THE finding of a single copy gene for rRNA in the micronucleus of the protozoan *Tetrahymena* by Yao and Gall (*Cell* **12**, 121; 1977) is the latest result of their investigation into the structure and organisation of the rRNA genes in this organism. It leads the authors to suggest that *Tetrahymena* may represent an evolutionary linkage between eukaryotes and their prokaryote-like ancestor.

Tetrahymena, a ciliated protozoan, contains a germinal diploid micronucleus which maintains the genetic continuity of the organism, as well as a polyploid macronucleus, which is formed after conjugation from the zygotic nucleus. The macronucleus functions as a somatic nucleus directing most of the cell's transcriptional activity during vegetative growth, but is destroyed at the end of each sexual generation. By DNA-RNA hybridisation using the 25S and 17S rRNAs of *Tetrahymena*, Yao *et al.* had previously shown that although 0.3% of the DNA in the macronucleus (which contains 23 times as much total DNA as the micronucleus) codes for rRNA (about 200 copies per haploid genome) only about 0.03% of the micronuclear DNA is rDNA (*Proc. natn. Acad. Sci. U.S.A.* **71**, 3082; 1974). Moreover, the vast majority of the rDNA in *Tetrahymena* exists as extrachromosomal pieces, about 13×10^6 daltons, each containing two copies of the rRNA genes arranged in a palindrome (Karrer & Gall, *J. molec. Biol.* **104**, 421; 1976; Engberg *et al. J. molec. Biol.* **104**, 455; 1976).

Considering the micronuclear origin of the macronucleus and the 10-fold difference in the rDNA content of the two nuclei, it is reasonable to suggest that the extrachromosomal rDNA of the macronucleus arises by amplification from one or a few rDNA copies integrated into the germinal chromosomes of the micronucleus. Working

from this hypothesis, Yao and Gall have tried through their experiments to establish the molecular basis of this amplification, asking whether or not the rRNA genes are in fact integrated into the micronuclear genome and if they are, do they have the same palindromic structure as in the extrachromosomal rDNA in the macronucleus, and furthermore are there any rDNA copies integrated into the macronuclear chromosomal DNA?

To answer the first question Yao and Gall digested macronuclear and micronuclear DNA individually with restriction endonucleases. *EcoRI* cleaves *Tetrahymena* rDNA at one site yielding two classes of fragment (10×10^6 and 1.6×10^6 dalton) from the extrachromosomal rDNA palindrome. Yao and Gall digested micronuclear DNA with *EcoRI* and identified the fragments containing rDNA sequences by radioactive cRNA probes. They found the 10×10^6 and 1.6×10^6 dalton fragments (probably as a result of unavoidable contamination with macronuclear rDNA) but also a third fragment of 5.3×10^6 daltons. They interpret this to be derived from one end of an integrated rDNA gene together with its flanking chromosomal sequences. This fragment appeared as a band of very low intensity suggesting that a very few copies at most of this integrated structure exist in the genome, most probably only one.

By analogous restriction enzyme mapping experiments with *BamHI* and *HaeIII* Yao and Gall show that in fact the *EcoRI* digestion of the integrated gene also yields a second fragment of 10×10^6 daltons. This was not recognised in the first instance because it comigrated in the agarose gel with the largest of the extrachromosomal rDNA *EcoRI* digestion products which contaminated the preparation. These two 10×10^6 dalton fragments are not identical, however. In extrachromosomal rDNA the fragment is palindromic so that digestion with additional restriction endonucleases is always symmetri-

cal. In microsomal rDNA subsequent digestion is not symmetrical, indicating that the 10×10^6 dalton piece includes only 5×10^6 daltons of rDNA, the other half being the piece of chromosomal DNA immediately adjacent to the integrated gene. Yao and Gall conclude that the rRNA gene integrated into the micronuclear genome of *Tetrahymena* exists as a single copy and not in the palindromic structure observed in the macronucleus nor in a tandem array.

To determine whether there are any integrated rRNA genes in the macronucleus the same digestion experiments were carried out on macronuclear chromosomal DNA as free as possible from contaminating extrachromosomal rDNA. No digestion products containing rDNA sequences (except for the bands attributable to the contaminating extrachromosomal rDNA) could be seen. Thus it is probable that no rRNA gene is integrated into the chromosomal DNA of the macronucleus.

These findings lead directly to the conclusion that the extrachromosomal rDNA palindromes of the macronucleus must be derived from the single integrated rRNA gene in the micronucleus. Yao and Gall propose a very plausible model for this synthesis and amplification assuming that there exists at the end of the integrated gene a palindromic sequence just long enough to allow branch migration to occur. This would provide a primer for the replication of the integrated rDNA sequence and the newly synthesised sequence could eventually be released from the chromosome as one folded DNA chain which could then replicate semiconservatively to generate the palindromic molecule observed in the macronucleus.

The finding of only one or a few chromosomally integrated rDNA sequences in the micronucleus of *Tetrahymena* is reminiscent of the low multiplicity of rDNA in prokaryotes and in the organelles such as mitochondria of higher eukaryotes; how-

ever, there is no other eukaryotic system in which rDNA has been found integrated in the nuclear chromosomes as a unique sequence.

But perhaps in the case of *Tetrahymena* this situation seems quite reasonable. The micro and macronuclei can be considered analogous to the germinal and somatic cells of higher eukaryotes except that, with the two nuclei occupying the same cell, the micronucleus does not have to direct the synthesis of ribosomes to support

itself. The macronucleus can supervise the household functions such as transcription and translation. Perhaps in higher eukaryotes where the somatic and germinal functions occur in separate cells it is necessary to have multiple rDNA genes integrated into the germinal chromosomes in order to achieve the rRNA synthesis needed to maintain the germinal cell. Hence, '*Tetrahymena* may represent an evolutionary linkage between eukaryotes and their procaryote-like ancestor.' □

Electron diffraction up to date

from A. Howie

A conference on Electron Diffraction was held at Imperial College, London on 19–21 September, 1977.

FEW fields can surpass electron diffraction in illustrating the disparity in the pace of scientific revelation and the more measured tread of technological change. The fiftieth anniversary of the subject, marked by the conference, proved to be a timely occasion to discuss the recent work which has at last realised the promise held out by the initial experiments. Although the two main active branches of the subject stem directly from the original low energy reflection experiments of Davisson and Germer and the high energy transmission experiments of Thomson and Reid, progress in both cases has been dependent on major instrumental technology such as the development of electron microscopes and ultrahigh vacuum equipment. As emphasised by T. Mulvey (University of Aston) in an entertaining review of the early work, formidable problems of specimen preparation had also to be overcome.

The high energy work is now concentrated in electron microscopy which has become a key discipline both in materials science and in biology and depends (as in the Abbe theory of the optical microscope) on the transmission diffraction imaging process. The diffraction contrast method, most widely used in materials applications, does not resolve the atoms directly but can provide images of crystal defects at a resolution of 1.5 nm (with weak beam methods) and an accuracy of 2×10^{-3} nm in the atomic displacements. It is particularly suitable for structures like metals with small unit cells and was described by Sir Peter Hirsch (University of Oxford) who concentrated on the detailed way in which

dynamical theory can explain the observations both qualitatively and quantitatively. Improvements in electron microscope performance have stimulated considerable activity in direct lattice imaging, discussed by J. M. Cowley (University of Arizona) with reference to the study of complex oxides and related materials with large unit cells. The information about the projected structure obtained in this way is of great significance in numerous chemical and mineralogical applications as well as in biology.

Despite the activity in real space imaging, all interest in the transmission diffraction pattern itself has not been lost, particularly for the study of diffuse scattering in highly disordered materials. In addition, the use of convergent beam diffraction patterns taken from small areas of crystalline specimens using focused illumination and pursued for many years by P. Goodman and colleagues at CSIRO Melbourne, has become a much more routine technique with better vacuum and other instrumental improvements. A major contribution to the meeting by J. W. Steeds and his team (University of Bristol) showed how local information about structure, composition and lattice parameter can be conveniently obtained from convergent beam patterns taken along crystallographic zone axes. It can be predicted that this technique will be increasingly popular, particularly in the context of scanning transmission microscopes where extremely small areas can be probed.

The increasing use of zone axis orientations, previously avoided because of the large number of diffracted beams excited, was perhaps the best evidence presented of the mounting confidence in the use of high energy diffraction theory in such situations. This has followed increases in computing power and efficiency enabling features of electron microscope images

to be interpreted more and more quantitatively with various plane wave formulations of dynamical theory but has also been helped along recently by several useful tricks borrowed from molecular and solid state valence electron theory. Indeed for the band theorist, high energy diffraction theory has emerged as an exciting new two-dimensional game with a ball of variable mass whose behaviour becomes classical in the limit of tight binding.

Electrons rarely scatter only once, particularly in the low energy electron diffraction (LEED) range. The development of a successful dynamical diffraction theory has therefore been even more crucial in the recent use of LEED for detailed studies of surface structure. Probably the key here was the recognition about 1969 of the importance of inelastic scattering described by an optical potential which is now fairly well understood over the whole energy range, even if some basic questions such as the treatment of thermal diffuse scattering or the use of the Doby Waller factor in LEED may require more study. S. Y. Tong (University of Wisconsin) gave an excellent survey of the recent results on surface structures. About 80 structures have now been determined including a considerable number of gas-covered surfaces so that the stage of looking for trends and general principles has now been reached. The fit between theory and experiment, though not quite perfect, is most impressive and generates considerable confidence that the structures deduced will be confirmed when tested by other methods such as X-ray diffraction, in the case of sufficiently perfect underlying crystals, or angle-resolved Auger emission and similar techniques which lean on LEED for their interpretation. More speculative topics on which some progress was reported were the use of spin-polarised LEED and of surface resonance effects when a diffracted beam is parallel to the surface.

Surface structure can also be studied by reflection high energy electron diffraction (RHEED) at glancing angles. J. B. Pendry (Daresbury Laboratory) described efforts to extend LEED theory to this case using the 'chain method' where scattering from atomic rows is treated as a cylindrical problem. The ultimate advantage of electron diffraction over X-ray diffraction, the ability to form real space images, can be pressed home much more easily at these energies, particularly using scanning electron microscopy. It looks probable, therefore, that yet another development in instrumental technology will eventually allow electron diffraction to extend its steady progress to the detailed study of real surfaces. □

Energetics and antibiotic uptake

from J. R. Saunders and
Venetia A. Saunders

ALTHOUGH the modes of action of most antibiotics are well established, much less is known of the processes by which such drugs are taken up by bacteria. In a recent paper (*Antimicrobial Agents Chemother.* 12, 163; 1977) Bryan and Van Den Elzen propose a mechanism to explain the transport of one particular group of antibiotics, the aminoglycosides.

The accumulation of streptomycin (or gentamicin) by bacteria can be divided into three phases. The initial phase is energy-independent and apparently involves ionic interaction of drug molecules with the bacterial surface. This is followed by an energy-dependent phase (EDP-I), which occurs before inhibition of protein synthesis by the antibiotic. In cells that are sensitive to streptomycin, but not those that are ribosomally resistant, this is followed by a third phase (EDP-II) which is also energy-requiring. This phase marks the onset of inhibition of protein synthesis and loss of cell viability and is characterised by a higher rate of streptomycin accumulation than EDP-I.

Bryan and Van Den Elzen have found that mutant strains of *Escherichia coli* deficient in haem production, ubiquinone production or active transport mechanisms accumulate less streptomycin or gentamicin than corresponding parental strains. In addition, such mutants show increased resistance to those and most other aminoglycosides. But they exhibit an increased susceptibility to the aminocyclitol antibiotic spectinomycin. This corresponds quite well with the fact that this drug is more effective than the aminoglycosides against anaerobic bacteria. Increased uptake of radioactive streptomycin was observed in a mutant that is unable to couple oxidative phosphorylation to electron transport (Unc⁻) and is also found defective in membrane-bound ATPase. It is interesting that this strain is hypersensitive not only to aminoglycosides but also to spectinomycin.

If streptomycin is transported into bacteria by means of a specific carrier system then compounds of closely related structure might be expected to compete with it. This is apparently not the case, but accumulation of

How many species?

from A. Hallam

A LIVELY controversy has grown up among palaeontologists in recent years about whether marine invertebrate species numbers, or richness, increased systematically through the Phanerozoic or whether an equilibrium situation has existed, with the apparent increase through time being an artefact. The leading advocate of the first view is J. W. Valentine, who has argued for an order of magnitude increase from the early Palaeozoic to the late Cainozoic, while the steady state alternative has been propounded principally by D. M. Raup.

Raup (*Paleobiology* 2, 289; 1976) estimated the number of species described for each of the geological periods by tabulating new species reported in the *Zoological Record*, and established a strong correlation between apparent species numbers and the present areal distribution of rocks per system. Now Sheehan (*Paleobiology* 3, 325; 1977) has come up with another interesting finding, having made a study of those palaeontologists expressing interest in fossils of particular systems, using data from the *Directory of Palaeontologists of the World*. It turns out that there is an excellent correlation ($r = 0.94$) between the number of carefully defined 'palaeontologist interest units' and the number of described species per geological period. Sheehan concludes that the total numbers of described species do not seem to reflect meaningful estimates of the original diversity.

In his reply to Sheehan, Raup acknowledges the good correlation established between the number of species described and the number of interested palaeontologists, as well as outcrop area, but maintains that nothing very positive can be inferred

from this. He prefers the view, though he cannot prove it, that geological systems with more available rock have more species and hence more species are described. In other words most palaeontologists are attracted to the most fossiliferous rocks!

Is there any way out of this impasse? Apparently there may be, provided a distinction is drawn between assemblages from different environments, which can be compared from period to period. This has been attempted by Bambach (*Paleobiology* 3, 152; 1977), who analysed data from hundreds of fossil assemblages from three different types of environment, as inferred from the facies. The high stress, marginal marine environment always has the lowest faunal diversity and the open marine environment the highest, while the variable nearshore environment has intermediate values. Bambach's data seem to indicate that within-habitat variation in species numbers is small for long intervals of time, and that the number of species has increased by a factor of about four since the mid-Palaeozoic, with variable nearshore environments showing a less pronounced increase than the open marine, while the high stress environments show no significant change. Thus Valentine seems to be partly vindicated, but substantial problems of interpretation remain, notably the factors controlling within-habitat species richness. Bambach speculates that changes through time of availability of food resources might be the most significant, but this is clearly an area demanding much further study.

A. Hallam is Professor of Geology in the University of Birmingham.

aminoglycosides in either whole bacteria or spheroplasts is antagonised in the presence of divalent cations. This could imply that these antibiotics are taken up by a carrier for such ions. Unfortunately no system capable of carrying a wide range of divalent cations has been described. A further significant finding is that calcium ions are less effective in inhibiting streptomycin uptake in a ubiquinone-deficient (*ubiD*) mutant than in the wild type. This indicates that ubiquinone has a direct role in the transport of this antibiotic. There is indeed a very good correlation between susceptibility to aminoglycosides and the possession of respiratory quinones. Thus obligate

anaerobes such as *Clostridium perfringens* which have no respiratory quinones are resistant to aminoglycosides. On the other hand, aerobic organisms possessing ubiquinone or menaquinone are usually sensitive to these drugs. Furthermore, aerobically grown cells of *E. coli* are many times more sensitive to these drugs than are anaerobically grown cells. This suggests that uptake of aminoglycosides is dependent on energy derived from aerobic metabolism.

A simple explanation for the data obtained would be that aminoglycoside transport requires carriers that are dependent on trans-membrane proton motive force. However, in the absence

J. R. Saunders is a Lecturer in the Department of Microbiology at the University of Liverpool, and Venetia A. Saunders is a Lecturer in Microbiology at Liverpool Polytechnic.

of an identifiable carrier for such antibiotics Bryan and Van Den Elzen propose an alternative hypothesis. They envisage that EDP-I represents transport of aminoglycosides across the cytoplasmic membrane by a process involving "the aerobic membrane energisation complex". The respiratory quinones probably act directly by binding streptomycin and transporting it across the membrane. A further but less likely possibility is that these quinones act indirectly by activating another component of the complex or a transport protein. EDP-II occurs

because membrane-bound ribosomes have a higher affinity than the transport system for streptomycin. Sensitive ribosomes consequently act as a sink for accumulating drug molecules. This would explain why EDP-II is lacking in strains which are ribosomally resistant to streptomycin. The exact mechanism of aminoglycoside uptake requires verification, but the insights provided by this work should help in the design of aminoglycosides and possibly other antibiotics with increased penetrability. □

Serum albumin structure and function

from Michael Geisow

ALTHOUGH serum albumin is one of the most familiar and abundant proteins, many aspects of its structure and function remain obscure, although knowledge of more exotic proteins has frequently reached the stage of detailed molecular models. In consequence it is pleasing to be able to report recent developments which give a clearer picture of this much studied, but poorly understood protein.

Roles traditionally assigned to albumin include the transport of fatty acids and other metabolites in plasma, although a host of minor functions continue to be suggested and discussed. Albumin is principally synthesised in the liver and recent work has brought its biosynthesis into line with that of other secretory products of cells. Albumin exists intracellularly with a six amino acid NH_2 -terminal extension. As in the case of proinsulin, this pro-form is actually made with a further NH_2 -terminal 'pre' peptide, which is removed at the sites of synthesis.

The impetus for much new work on albumin stems from the complete sequences of bovine (581 amino acids) and human (582 amino acids) albumins. These are the considerable achievements of J. Brown at Texas University

and B. Meloun of the Czechoslovak Academy of Sciences. The primary structures of other animal albumins are well under way. A striking feature of these sequences is the repeating double-loop motif formed by crosslinks from adjacent cysteine residues (Figure 1). These define three domains or homologous regions of chain (marked I to III in Fig. 1). There are strong amino acid sequence similarities between these domains.

It has become usual to assume that homologous regions of polypeptide form closely similar three-dimensional arrangements. This assumption, together with the considerable folding restrictions imposed by 17 disulphide bridges, has led Brown to construct a plausible model for the albumin domain (11th FEBS Meeting abstracts). His conceptually simple structure consists of six α -helices (three double loops) which form a roughly cylindrical domain. Albumin is made up of an association of three such cylindrical domains.

The obvious repetition of structural features has led to proposed evolutionary pathways for albumins (Brown *Fedn. Proc.* 35, 2141; 1976; McLachlan & Walker *J. molec. Biol.* 112, 543; 1977). Both groups agree that the modern protein descended from duplications of a single double loop struc-

ture. Brown suggests from implied structural evidence that myoglobin and albumin share a common ancestor. McLachlan on the other hand believes that this homology results from convergent evolution of albumin and the globins.

Whatever the ancestry, it seems relevant to ask what function the primitive albumin fulfilled, since the much larger modern protein must represent a sophistication of the original idea. Some answers to this question may have been supplied already by the study of the properties of albumin fragments. For some time workers have known that limited proteolytic treatment of native albumin produces large fragments, evidently by cleavage at unstructured parts of the molecule. The recent sequence information has enabled albumin fragments to be accurately located and released these studies from relative obscurity. Many workers, particularly from T. Peter's group at the Mary Imogene Basset Hospital, New York, have found that individual domains or series of double loops result from mild enzyme treatment. Such fragments are well ordered and may largely retain the conformation they assumed in the intact albumin molecule (Reed *et al. Biochemistry* 14, 4578; 1975). Even more significantly, fragments retain binding sites associated with the native protein, so it is now possible to allocate these to particular regions of the sequence. On this evidence it seems very likely that the primitive albumin, a double loop, provided a useful binding function in some primordial plasma. Perhaps the evolutionary pressure to extend the number of double loops was strong, not merely to bind other ligands, but also to prevent losses from an equally primitive kidney!

Brown's domain model provides an idealised concept of an albumin-binding site. The cylindrical centre of each domain is a space into which project hydrophobic side chains from the helices. The entrance to this space, formed by the tips of the long disulphide loops in Fig. 1 is lined with positive charges. Lysines and arginines seem to cluster at these points. Such a hydrophobic cavity with localised positive charge is tailor-made for the organic anions which occur so frequently as albumin ligands. It is quite certain that the basic side chains in this site model become labelled when reactive ligands are bound to albumin (Gambhir *et al. J. biol. Chem.* 250, 6711; 1975). Interestingly, albumin domains seem to have developed some functional specialisations. Fatty acids bind most avidly to domain III while bilirubin and other aromatic ligands prefer domains I and II. Such close structural homology, yet diverse bind-

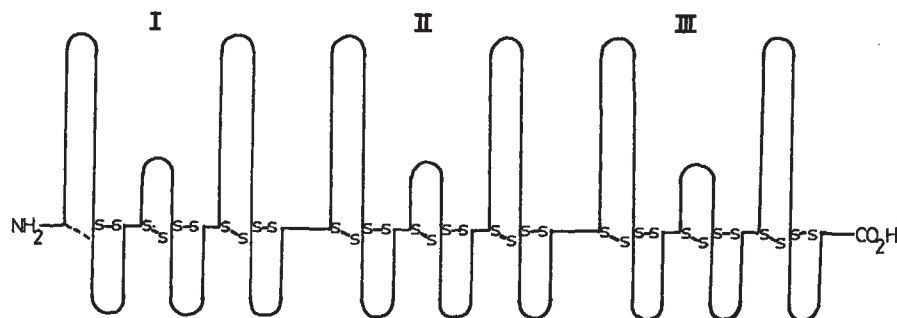


Fig. 1 The double-loop structure of serum albumin.

Michael Geisow is a member of staff at the National Institute for Medical Research, London.

ing properties, calls to mind the diversification of the immunoglobulin domains.

Albumin recently joined the list of allosteric proteins, with reports that its binding sites were not independent. The binding of a spin-labelled dinitrobenzene is enhanced by 80% when fatty acids are added to albumin and spin label. The spectrum of the bound label also changes, indicating an alteration of its protein environment (Soltys & Hsia *J. biol. Chem.* **252**, 4043; 1976). The affinity of bilirubin for albumin is also increased upon fatty acid binding (Broderon *11th FEBS Meeting*). Arthur Spector reported at the same meeting that fatty acid, presumably bound to domain III, affected spectral properties of an amino acid solely found in domain II. Some caution is still required, since at moderate concentrations most albumin ligands bind at more than one site and it may be difficult to distinguish between cooperative effects and a more simple direct competition. However, Broderon, Torgny and Sjöholm (*J. biol. Chem.* **252**, 5067; 1977) have

shown that albumin does independently bind the antianxiety drug diazepam and bilirubin. These two do not share the same site and the calculated coupling between their sites (expressed as free energy) is less than 50 cal mol⁻¹.

Seen in terms of the domain model of albumin, cooperative phenomena are interesting because they provide a readily available model for studying information transfer between discrete parts of the same polypeptide chain. In a clinical context, these studies are important because they imply that natural ligands and common drugs do not necessarily interact with albumin independently or in a simple competitive sense. Since some drugs are strongly bound by albumin, binding is an important factor in dose levels. The recent results suggest that the free concentration of such drugs could be markedly altered by the dietary fluctuation of plasma fatty acids. It is to be hoped that some of the current speculations and the now huge body of pharmacological measurements connected with albumin can soon be integrated. □

products are continually required for differentiation away from mesothorax.) These mutants produce transformations in the entire compartment normally requiring the gene product.

P. A. Lawrence (MRC Laboratory of Molecular Biology, Cambridge) described evidence that some segments are further subdivided into anterior and posterior compartments; here the engrailed gene product is apparently required exclusively for the posterior cells. The gene product is essential for the proper development of the posterior compartments and for the precise delineation of the boundary between anterior and posterior cells. He described unpublished work of G. Struhl (MRC, Cambridge) on the proboscis which showed that it is divided into anterior and posterior compartments and also dependent on the engrailed gene in a homologous way to the thoracic segments.

Another mutant which can be viewed within the same conceptual framework is the mutant *transformer* in which XX genetic females are made into almost perfect males.

R. Nöthiger (University of Zurich) described experiments which demonstrated that all embryos contain two separate primordia, one for male and one for female genitalia, each sex containing one 'silent' primordium. By making large *transformer* clones in an otherwise wildtype female. Nöthiger was able to conjure up a complete male set of genitalia in an intact female; *transformer* clones in the female primordium produced loss of part of the female genitalia.

The picture emerging from these four talks is one of a series of 'master' genes, which are activated in particular places, and which control the development of precisely defined pieces of the insect.

At the cellular level, O. Siddiqui (Tata Institute, Bombay) has studied the development of the *Drosophila* nervous system in a range of chemosensory mutants some of which show temperature sensitive development of the sensory pathway. The development of the nervous system was also the subject of a talk by J. White (MRC, Cambridge) who has used mutants and laser microsurgery to investigate the pattern of cell lineage in the ventral nerve cord of nematodes. Removal of individual cells by laser revealed that inductive interactions can occur, and that the vulva precursor cells can regulate their proliferation to compensate for the experimental elimination of specific cells. One alternative to conventional genetic approaches to the study of development is the use of cell-free systems, two of which were described at the meeting. R. Laskey

Genes in development

from Ron Laskey, Peter Lawrence and Eddy De Robertis

A conference on Developmental Genetics was convened by the Accademia Nazionale dei Lincei and held in Rome on October 31–November 1, 1977.

PARTICIPANTS in two days of talks held at the Accademia Lincei in Rome—'the oldest scientific foundation in the modern world', tried to tackle the ancient problem of how eggs plus genes produce animals. It was symptomatic of the renewed interest in *Drosophila* that the whole of the first day was devoted to that fly. E. B. Lewis (Cal-Tech) revealed some exciting new results in the bithorax system, the complex genetic locus responsible in part for the segmented pattern of the larva and adult fly. The scanning electron microscope showed up some hitherto unremarked distinctions between the different segments in the newly hatched larva so that the segmental patterns of new mutations and deficiencies could be studied. Flies deficient for the whole of the bithorax

complex developed only as far as the first stage larva, which consisted entirely of mesothoracic segments. Further analysis of partial deficiencies and 'constitutive' mutants indicated a group of new genetic functions (to the right of *pbx*) which Lewis called *polythoracic* and which are apparently necessary for the normal differentiation of the abdominal segments—all regarded as structures which diverge from the archetypal mesothorax.

A. Garcia-Bellido (Madrid University) pursued the problem of how the genes of the bithorax system are switched on in particular places. In his view the bithorax system responds to positional information in the egg; a process which can be interfered with in at least three ways: (1) the positional information can be disturbed, for instance by ether treatment of the blastoderm-stage egg. This results in groups of cells developing faithfully into a portion of inappropriate segment; (2) the same phenotype can be produced by certain mutations (*Rgpbx*, *Rgbx*) in genes which might produce regulatory molecules involved in the response of the bithorax system to the positional information; (3) mutations in the bithorax system itself (whose

The authors are at the MRC Laboratory of Molecular Biology, Cambridge.

(MRC, Cambridge) described a *Xenopus* egg system in which chromatin assembly takes place. Each egg can convert an amount of DNA equivalent to 6000 nuclei into regularly spaced nucleosomes in the absence of DNA synthesis. Laskey has found that all the materials required for nucleosome assembly cofractionate in a single particulate fraction, suggesting that histones are organised into a nucleosome precursor complex before they bind DNA.

G. Tocchini-Valentini (CNR, Rome) described a *Xenopus* oocyte system which elongates and terminates replicating chromatin. But neither he nor Laskey could find evidence for initiation of DNA replication *in vitro*, although it can apparently be seen in micro-injected eggs.

The fusion of genetic and molecular approaches to the study of development provided a stimulating two-day meeting. □

Problems with pollen

from Peter D. Moore

PALYNOLOGISTS studying sediments of Quaternary origin have certain advantages over colleagues concerned with older materials for there is a higher likelihood that both the taxa and the plant communities with which they deal remain extant. There are exceptions, of course, such as the tundra communities which occupied low latitudes and low altitudes in the periglacial conditions obtaining at the close of the last glaciation. One cannot assume that tundra communities of modern high latitudes or high altitudes have the same composition. But generally the Quaternary palaeoecologist uses the present as a Rosetta stone by which to interpret the past.

Reconstruction of the past mosaic of plant communities on the basis of a subfossil assemblage of pollen grains is currently being greatly assisted by accumulating information concerning modern pollen rain from contemporary plant communities. It is remarkable that for many decades palynologists have been content to interpret their fossil data without such information. Fortunately, however, indications from most modern pollen rain studies have confirmed the broad correlation between vegetation and its pollen output which has always been assumed. The work of Lichti-Federovich and Ritchie (*Rev. Palaeobotan. Palynol.* 7, 297;

1968), for example, on surface lake sediments in central Canada, showed that the major forest belts of the region could be distinguished on the basis of their pollen spectra. Certain anomalies were evident, such as the transport of spruce pollen into the treeless tundra areas of the north, but there was generally an acceptable agreement between vegetation and pollen rain.

Such studies have been chiefly concerned with the major components of both vegetation and the accumulating pollen assemblage, but detailed interpretation of palaeodata may also depend on the presence of relatively small quantities of a taxon with narrow ecological or phytosociological limits. It is often possible to infer the presence of certain vegetation types and hence certain habitats and microenvironments on the basis of the occurrence of these 'indicator types.' In this respect the work of Janssen has been of great value (for example *Ecol. Monogr.* 37, 145; 1967). He has used the technique of comparing vegetation transects along environmental gradients with the pollen accumulating along them; in this way it is possible to observe how closely certain pollen types are associated with particular plant communities, even when their overall representation in the pollen spectrum is small.

On the basis of Janssen's work it is evident that pollen samples taken from the surface sediments of a water body will differ quite considerably from those in adjacent reed swamps or woodlands. So when interpreting fossil data, the nature of the sediment under consideration should be taken into account. One further aspect of this type of study which still lacks detailed documentation is the degree to which surface samples of the same type and under the same vegetation vary in their pollen composition. Is it possible that the variation within certain plant communities is greater than that between communities? In many surface pollen studies it has not been possible to glean this type of information because duplicated samples have been bulked before analysis, but a recent study of pollen rain in Alaska by Birks (*Can. J. Bot.* 55, 2367; 1977) now provides the necessary information for certain vegetation types.

Birks distinguished four major vegetation types, *Picea glauca* forest, which contained *Betula glandulosa* as a shrub layer, *Betula glandulosa* dominated shrub-tundra, *Populus balsamifera* stands within the forest, and open tundra vegetation dominated by *Dryas integrifolia*. In addition to the detailed description of these vegetation types, Birks collected samples of moss polsters, surface muds from lakes and superficial peat samples from sedge swamps from each of the plant com-

munities. A visual inspection of the data resulting from the analysis of these samples suggests that certain pollen characteristics are specifically associated with certain vegetation types. For example, only in samples originating from the *Populus*-dominated stands does the *Populus* pollen type occur at levels greater than about 2%, and only in the *Dryas* tundra does the level of *Dryas*-type pollen exceed about 2%.

Birks has subjected the pollen data to principal coordinates analysis, in which the pollen samples are ordinated with respect to one another on axes which represent dissimilarity. On one axis he shows a polarisation of the *Populus* forests and the *Dryas* tundra with the *Picea* forests and shrub-tundra forming an undistinguishable group in the centre. On the other axis the extremes are occupied by swamp peat samples and moss polsters respectively. One of the most important criteria which may account for this latter separation is the relative proportion of Cyperaceae pollen in the samples. One must conclude from this analysis that the degree of variation found within both the *Picea* forest and the shrub-tundra samples are such that the two vegetation types cannot be separated on the basis of their pollen spectra. This being so, one would not be able to differentiate between them in subfossil pollen assemblages.

This analysis was based only on those pollen types having a relative abundance of 5% or more of the total pollen sum in at least one of the samples; in other words, only the major pollen types are used for differentiation. There remains the possibility that indicator types of lower abundance could be used. Inspection of the pollen data suggests that this is unlikely to be a particularly helpful approach. Only one terrestrial herb taxon, *Astragalus*, is found in a single *Picea* forest sample but in none of the shrub tundra samples. On the other hand there are ten such taxa found scattered among the shrub-tundra samples which are not found in the spruce forest sites. As a final straw at which to clutch, one could compare the overall herb pollen diversity in the two sets of samples. Inspection indicates that the shrub-tundra has a generally larger component of low abundance, herbaceous taxa, possibly reflecting the open vegetation. A comparison of the two sets of data using a diversity index such as the Shannon-Wiener function might provide a means of resolving these two communities on the basis of their pollen spectra. Meanwhile, the fact that such problems can arise in the separation of modern vegetation types should encourage caution among those who seek to interpret fossil assemblages. □

Peter D. Moore is a Senior Lecturer in the Department of Plant Sciences at King's College, London.

review article

Applications of proportional chambers and drift chambers in high-energy physics and other fields

G. Charpak*

Proportional counters, which were superseded in high-energy physics by developments such as spark and bubble chambers, have recently undergone modifications which have considerably increased their space and time resolution. These modified counters are finding applications not only in high-energy and nuclear physics but also in biology and medicine.

PROPORTIONAL counters were an essential tool in nuclear physics until the beginning of the 1950s. Because of their practical importance their properties were extensively investigated and to a large extent well understood¹. The powerful properties of structures different from the simple cylindrical shape were overlooked, however, and as we shall see, the multiwire structures permit space and time resolutions that are orders of magnitude better than those of cylindrical structures.

The counters were used for many purposes: counting of ionising radiations, measurement of their energy, localisation with an accuracy, both in space and time, usually set by the counter size. They have played only a minor part in the development of nuclear or sub-nuclear physics in the last quarter of a century, when they were superseded by the scintillation counters, faster and more versatile, the nuclear emulsions, the spark chambers of various types localising the position with accuracies better than a millimetre; the bubble chambers, visualising spatial configurations in particle reactions of an incredible complexity, and many other detectors.

A recent development², based essentially on the same principles as the cylindrical counter but making a proper use of multiwire structures, is, however, making a radical change in the experimental strategies in low-energy and high-energy physics, and has also begun to find applications in several other fields, such as crystallography and medicine.

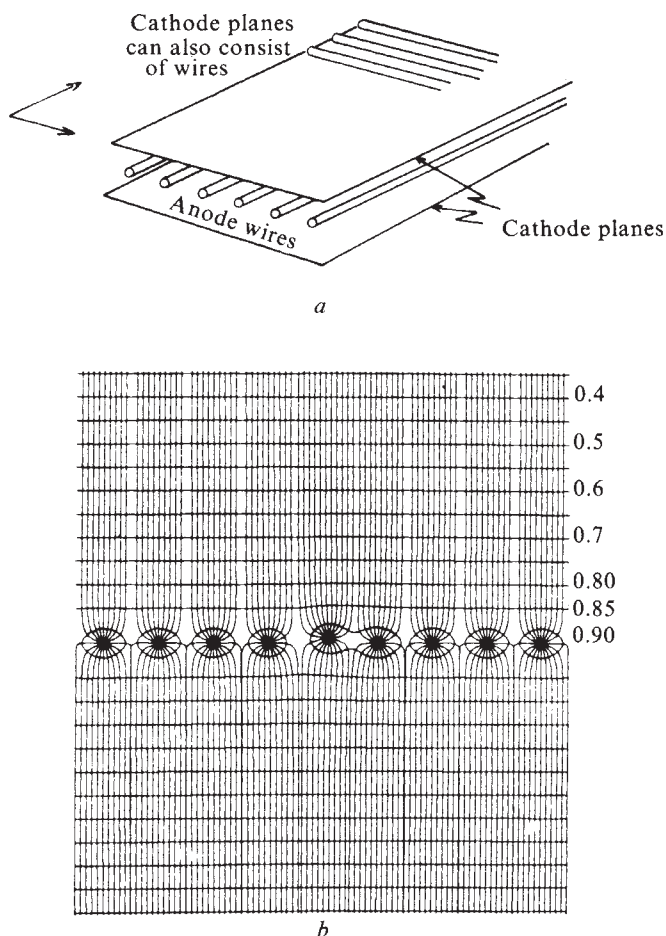
Ordinary cylindrical proportional counters

Since this basic instrument is described in many text books, let us mention only its most salient features. It is typically made of a cylindrical cathode with a thin wire anode at its axis. The gas filling usually consists of a noble gas mixed with organic molecules. In such a structure the electrical field decreases as the inverse of the distance r to the axis. In the region close to the wire, the field is so intense that free electrons acquire enough energy in a mean-free path to ionise the molecules of the gas, thus leading to an avalanche of electrons localised close to the surface of the wire. A typical collision mean-free path at atmospheric pressure is $1\ \mu\text{m}$. The maximum size of an avalanche is around 10^6 electrons. For gains exceeding this limit, dependent on the gas and geometry, the ultraviolet photons emitted by the excited atoms induce a propagation of the discharge. The radial extension of an avalanche at atmospheric pressure is thus close to $20\ \mu\text{m}$ only.

Since the process of amplification leading to the detectable pulse is produced after the ionisation electrons have reached the region close to the anode, the time of appearance of the pulse will depend on the initial position of the electrons, and will vary with the position of

the particle. This introduces an uncontrolled jitter in the timing of the particles, which reaches a few hundred ns for 1 cm diameter. This is one of the reasons why these counters were superseded by the scintillation counters, which, although more expensive, have a 100 times better resolution.

Fig. 1 A multiwire proportional chamber: *a*, schematic view showing the cathode planes closely spaced around anode wires; *b*, electric field equipotentials and field lines in a multiwire proportional chamber. The effect on the field of a small displacement of one wire is also shown.



*CERN, Geneva, Switzerland

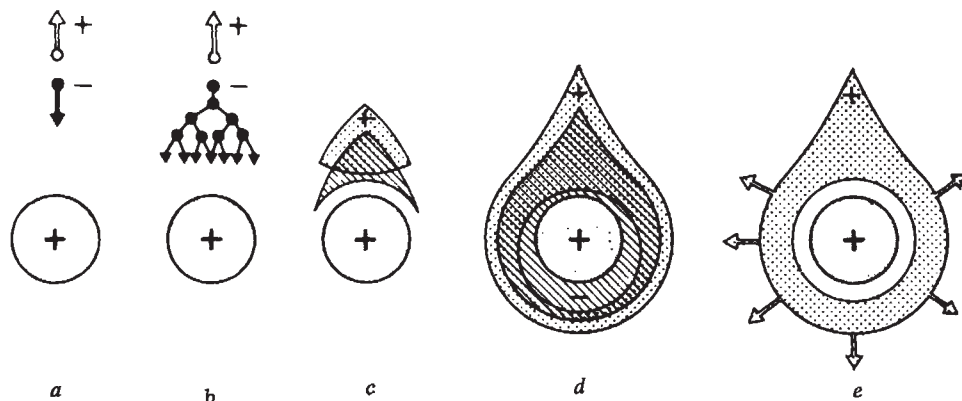


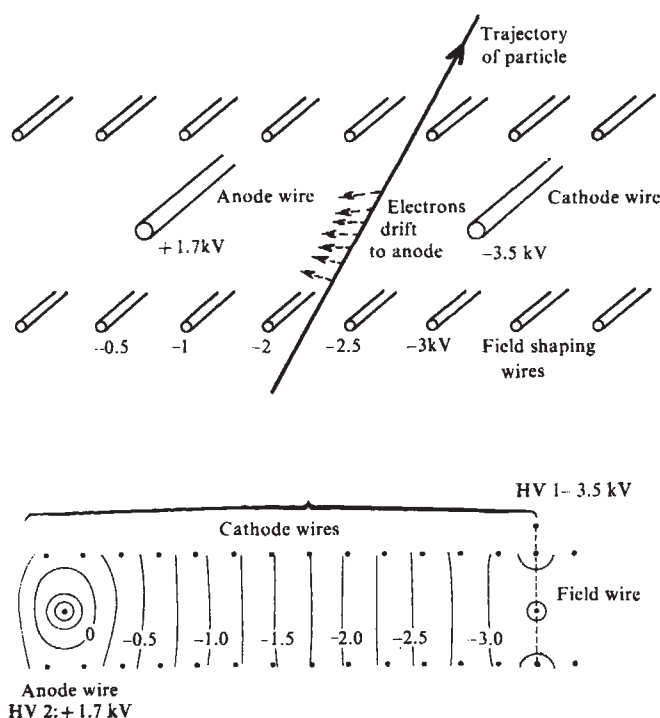
Fig. 2 Time development of an avalanche in a proportional counter. A single primary electron proceeds towards the anode (a), in regions of increasingly high fields, experiencing ionising collisions (b); owing to the lateral diffusion and photoionisation, a drop-like avalanche (c), surrounding the wire, develops. Electrons are collected in a very short time (1 ns or so) (d), and a cloud of positive ions is left, slowly migrating towards the cathode (e) Whether the cloud surrounds the wire or not, depends on the nature of the gas.

We shall see that by giving up the cylindrical structure, two steps have been accomplished: both the time and position of the particles can be measured with a high accuracy.

Multiwire proportional chambers (MWPC)

The electrical structure of the chamber comprises thin, parallel, and equally spaced positively charged wires, sandwiched between two cathode planes. Figure 1 shows the electric field potentials in such a structure. We see that in the region close to the wire the electrical field is exactly the same as in a cylindrical structure. In other words, as soon as an electron liberated in the gas by an ionising radiation has reached the region of multiplication, it proceeds exactly as in a cylindrical counter. Although the wires are independent for the multiplication process, one may wonder whether their capacitive coupling does not short-circuit them and thus reduce them to a single electrode when they are as close as 1 mm. Indeed, when an external fast pulse is fed to one wire we observe the propagation of the pulse with the same sign, from wire to wire. This is not the case, however, with a pulse generated by the amplification process, and here we have to look into the detail of the pulse mechanism to

Fig. 3 Principle of construction of the adjustable field multiwire drift chambers: a, schematic view; b, equipotential lines. Cathode wires are connected to uniformly decreasing potentials, starting from ground in front of the anode. Field wires reinforce the field in the transition region to the next cell.



understand some of the properties which are responsible for the success of this type of structure.

Figure 2 shows the time development of the avalanche started by a single electron around a wire. Within a time shorter than a nanosecond, the avalanche results in a thin cloud of positive ions close to the wire, usually in a non uniform way. It was at first believed that the avalanches surround the wire. More recent experiments show that this is not usually the case and that this hypothesis is not required to explain the positive pulses on all electrodes neighbouring a sensing wire.

It is important to realise that the collection of the electrons does not usually give rise to a detectable pulse: it is too fast and too small. Its size is proportional to the potential drop experienced by the electrons while travelling in the gas, which is negligible since they travelled only a few μm . It is the motion of the positive ions that is responsible for pulses with the following characteristics. (1) Pulses are negative in the wires but positive in all surrounding electrodes. This is one essential feature: the neighbouring wires detect a positive pulse. Thus, the active wire is easily distinguished from its neighbours. It suffices to use amplifiers that are sensitive to one sign. (2) The pulses are fast-rising. The field is so intense close to the wire, usually above 10^5 V cm^{-1} , that rise-times of the order of 10 ns are observed at the beginning of the pulse, which then slows down and lasts for μs .

The important properties of a MWPC are as follows. (1) The space resolution is apparently dependent on the wire spacing since every wire behaves as an independent counter. In practice, 1 mm wire spacing is common for small structures (20 cm), 2 mm for large structures (200 cm), and chambers of $5 \times 5 \text{ m}^2$ are being operated with 3 mm wire distance. (2) The space resolution of a chamber can be much better than the wire spacing. The coordinate of the avalanches along the wire can be measured by different methods. Accuracies better than $100 \mu\text{m}$ can be obtained. Since a two-dimensional read-out is essential for the detection of neutral radiations, I shall discuss the recent progress in such measuring methods in connection with some applications. (3) The time resolution is also dependent on the wire distance. The closer the wire spacing the shorter is the time taken by the electrons liberated in the gas by an ionising particle to find a nearby wire to be detected. Practically 30 ns resolution is obtained with 2 mm wire spacing. (4) The maximum counting rate is limited locally by the space charge created by the positive ions. The chambers show a loss of gain at rates of the order of $10^4 \text{ events s}^{-1} \text{ mm}^{-2}$. Rates of several million counts s^{-1} are common in the practical use of the chambers.

Drift chambers

The time lag between the appearance of a pulse and the distance to the wire of the ionising track can be used to measure this distance^{2,3}. Typically it takes 200 ns for an electron to drift 1 cm in the chamber. This has given rise to an important class of detectors: the drift chambers. Measuring the time is very simple. Chambers with very large wire spacings can be used to make economical, large-size detectors. Figure 3 shows the structure of such a chamber. One example is given by the chambers built at Daresbury for an experiment at CERN: size 3.5 m, wire distance 12 cm, accuracy better than $350 \mu\text{m}$ throughout the active area⁴. The intrinsic

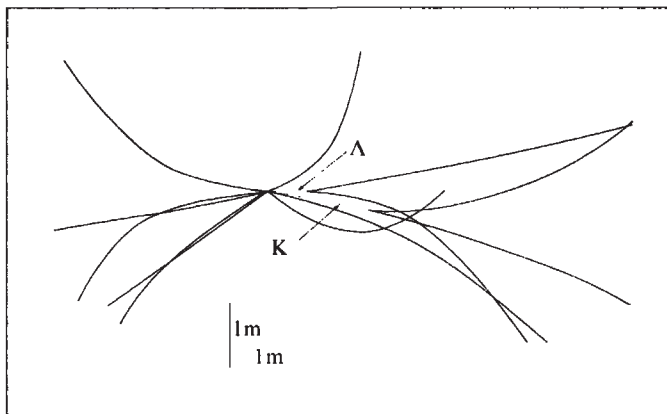


Fig. 4 Computer display of a high-energy interaction viewed by a system of multiwire chambers. At an intersection of the CERN proton storage rings, a system of 70,000 wires in a magnetic field detects the coordinates of the particles from one interaction. Typical rates: 150,000 interactions s. Background: 10^6 s^{-1} chamber, resolution time $\sim 100 \text{ ns}$.

accuracy is of the order of $50 \mu\text{m}$, as shown in smaller chambers used in experiments³.

Applications of MWPC and drift chambers

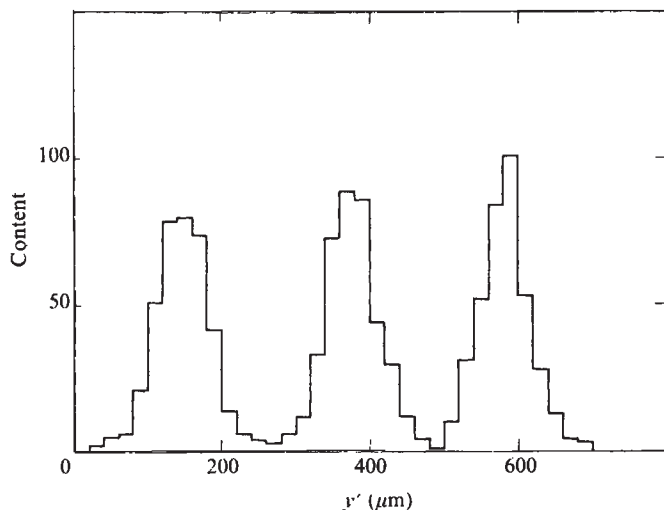
MWPC and drift chambers are now routine instruments in high-energy physics. They have in most cases replaced the spark chambers and sometimes the scintillation hodoscopes. They permit the detection of very rare events, for instance, in the hunting of new particles immersed in an enormous background of common reactions. The high time resolution and space resolution are the essential properties for this research.

Very large systems are in operation. Typical in this respect is the large detector equipping one intersect of the CERN storage rings. Set in a gap of about 10 m length, 1 m height, and 2 m width, chambers with wires of 1 m or 2 m length detect the trajectories of the particles produced in the head-on collisions of two 30 GeV protons. Figure 4 shows a complex event reconstructed by a computer from the data collected by 70,000 wires, 2 mm apart.

Many bubble chamber systems now use external systems of MWPC to give supplementary data on the particles emerging from the chambers.

Chambers are also now commonly used as detectors in nuclear physics in the focal plane of magnetic spectrometers, where

Fig. 5 Accuracy along the wire. Histograms of the coordinates of the charge centroid along a wire for three positions of a collimated beam with $200 \mu\text{m}$ separation (ref. 6).



accuracies of a fraction of a millimetre over lengths of the order of 1 m are easy to attain. Typical in this respect is the detector developed at the Massachusetts Institute of Technology⁵, where an accuracy of $130 \mu\text{m}$ (2σ) and an angular resolution of 17 mrad (2σ) is obtained for relativistic electrons in the focal plane of a spectrometer.

Outside physics, MWPC are now being applied to problems of localisation of X rays and neutrons in medicine. These radiations produce in the gas low-range ionising secondaries which do not emerge from the chambers. It is essential to localise the coordinates of the avalanches from a single gap. Since the very beginning of the MWPC, the groups working in the field of neutral radiations have designed various analogue methods to measure the coordinates of avalanches along the wires^{2,6}. They are based on the propagation of the signals in delay lines or resistive electrodes. A more recent development⁷ illustrates the considerable accuracy that can be reached both along the wire and in the interpolation between wires. It is based on the measurement of the centroid of the pulses induced on cathode strips. Figure 5 shows the positions of three 1.5 keV X-ray beams, spaced about $200 \mu\text{m}$ apart along the wire. A resolution of $\sigma = 35 \mu\text{m}$ is obtained, of which only $7 \mu\text{m}$ is contributed by the measurement, the remainder corresponding to the physical size of the charge distribution. The surprising thing is that this method gives also the position of punctual charge distributions, characteristic of X-ray secondaries, between the wires. It was for a long time postulated that the accuracy of a chamber is limited by the wire spacing. This is wrong in two cases: if the charges are distributed among several wires, the centroid method will give the interpolation; and if the charges are punctual, the centroid method will give the azimuth of the avalanche on the wire with an accuracy of about 5° for 2 mm wire spacings. This is illustrated by the radiograph of the word CERN⁷, cut out in a copper mask with letters of $50 \mu\text{m}$ width, 1.5 mm height. Although the wire distance is 2 mm, a continuous response is obtained with all the avalanches occurring on the wire centred on the letters only (Fig. 6).



Fig. 6 Radiograph of the word CERN. Dimensions of the word: 1.5 mm $\times$ 4 mm. Avalanches on only one wire: 2 mm wire spacing⁷.

This method will certainly be widely used in all problems where the two-dimensional read-out is important.

The most promising application of the chambers so far seems to be in the detection of X rays^{8,9} or neutrons^{10,11} diffracted by crystals made of large molecules, or by organic tissues with a regular structure¹².

At CERN, a spherical drift chamber has been developed¹³ to be used with the synchrotron radiation with the Orsay electron storage rings. Its present characteristics are: an angular aperture of 90° , an angular accuracy of 2.5 mrad, an efficiency of 100% for 8 keV photons, and a possible ultimate rate of 10^6 s^{-1} . A gain of at least 1,000 in the speed of data-taking is expected with respect to the conventional detection methods used with diffractometers. This opens up new fields of research, for instance in the study of quickly evolving structures.

Nuclear medicine also has problems of imaging, chiefly in the detection of X rays that are much more energetic. The minimum is the radiation of 27 keV emitted by ^{125}I , commonly used in labelling the thyroid tissues. The main interest, however, lies in radiations well above 100 keV, where the chambers are quite inferior to the widely used scintillation cameras in two respects: the low efficiency, and the poor spatial resolution due to the large range of the

secondary electrons in the gas. So far this has limited the applications to fields where radiations below 100 keV are of interest. Typical in this respect is the work undertaken at Rutherford Laboratory¹⁴, where a chamber measures bone densities with the 42 keV X rays emitted by ²⁵³Gd. The localisation of the X rays can be better than with NaI cameras¹⁵ and they cost far less.

A possible way of overcoming the low density of the gas is to use a drift space filled with suitable converters. Several groups^{16,17} are at present actively developing this approach. Efficiencies of up to 15% with accuracies of a few millimetres can be reached at 500 keV.

These chambers suffer from two drawbacks: no energy resolution, and a poor resolution time close to 0.5 μ s. They find a possible important application for positron cameras. The correlation of the two annihilation γ -rays give such a good selection of non-scattered γ -rays that it compensates partially for the lack of energy resolution. The poor time resolution may be a fatal obstacle to the use of this technique for medical applications. Present positron cameras based on NaI counters can operate with 10-ns resolution times. They lead to such expensive instruments, however, and the technique of positron imaging could be so powerful with good, cheaper detectors, that it is still worth making a thorough investigation of the possibilities offered by MWPC, which are better suited to large size, low cost detector systems than are the NaI cameras.

Conclusions

Multiwire chambers and drift chambers offer an interesting example of a technology developed in high-energy physics laboratories with a fair chance to find important applications in fields remote from high-energy physics. Whenever methods require the measurement of the position of ionising radiation, a possible application of these detectors can be found; this offers a good opportunity to make use of the huge effort in capital and skills invested by experimental groups working in high-energy physics.

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articles

Past volcanism revealed by Greenland Ice Sheet impurities

C. U. Hammer

Geophysical Isotope Laboratory, University of Copenhagen, Haraldsgade 6, DK-2200 Copenhagen, Denmark

Past volcanic aerosols have left Greenland Ice Sheet layers with elevated specific electrical conductivities that can often be used to estimate the magnitude of individual eruptions. Records of past volcanism may, therefore, be extended and help solve the problem of possible climatic correlation.

PRECIPITATION falling on the two large ice sheets in Antarctica and Greenland is extremely pure^{1,2}. The low background impurity level makes polar glacier ice suitable for investigating past violent volcanism in the form of additional impurities deposited shortly after the eruptions and stored in the ice.

Disregarding gases and organic material³, the deposition of impurities on the Greenland Ice Sheet is of the order of 50 kg km⁻² yr⁻¹ in times of low volcanic activity⁴. Assuming that the mean aerosol residence time in the upper troposphere is approximately 1 month⁵, the above figure corresponds to a background aerosol load of the order of 10⁹ kg in the upper northern hemisphere troposphere, which is close to an estimate based on Mauna Loa data⁶. Aerosols of volcanic origin may be detectable as increased aerosol fall-out, if their contribution to the upper tropospheric aerosol load is significant relative to the above mentioned background.

Violent volcanic eruptions inject into the atmosphere large quantities of impurities, part of which significantly reduces the

atmospheric transmissivity on a global or hemispheric scale up to three years after the eruptions. For example, the Krakatau (1883) and the Katmai (1912) eruptions caused a 20% reduction of the Northern Hemisphere atmospheric transmissivity by each injecting 16 $\times$ 10⁹ kg aerosol material into the northern stratosphere⁶ (Krakatau probably injected a similar amount into the southern stratosphere as well). Assuming that this material is deposited within 1 or 2 yr, the average volcanic contribution to the total upper northern tropospheric aerosol load in these periods was probably of the same order as the non-volcanic background (10⁹ kg).

Therefore, in spite of the spreading of the eruption cloud and the complicated deposition pattern caused by non-uniform wash-out by precipitation, any 'clean-air' location must be expected to receive significantly elevated amounts of aerosol fall-out in periods of high volcanic activity, at least in the same hemisphere. The most favourable areas for tracing past volcanism are the high altitude regions of the two large ice sheets, where the background concentration of non-volcanic aerosols is low. The Greenland Ice Sheet has the additional advantage of being fed by relatively frequent, heavy snow falls. The impurity concentration in the snow is proportional to the temporary aerosol load in the air⁷ and since the snow is accumulated with little melting and no run-off, analyses of absolute-dated Greenland cores provide a record of atmospheric turbidity, essentially above $\sim$ 2,000 m altitude.

None of the impurity nor its constituents has so far been shown

to be a specific volcanic tracer. Previous analyses⁸ performed on an ice core from Dye 3 (Fig. 1), indicated that most solid micro-particles above 0.4 μm radius are continental surface dust, even during periods of high volcanic activity. But the high sulphate content in the stratosphere shortly after recent violent eruptions suggests that most of the volcanic material in the stratosphere consists of sulphate, originating from the release of H_2S and SO_2 (ref. 9). Hence, elevated concentrations of H_2SO_4 and/or $(\text{NH}_4)_2\text{SO}_4$ might be detectable in Greenland snow up to three years after the volcanic event. Impurities, particularly of sulphuric acid, greatly influence the specific electrical conductivity, σ , of the melted ice, and as we analysed thousands of samples, this simple parameter was chosen as an indicator of volcanic activity. Of course, σ profiles cannot replace the comprehensive chemical analysis program that has commenced¹⁰, but they do reveal important characteristics of volcanic fall-out and may be used as a simple means of showing which of the numerous core increments are promising objects for detailed geochemical analyses.

Volcanic impurity concentration index

Using σ as a volcanic activity indicator implies a comparison of σ -profiles along absolute-dated ice cores with historical records of the volcanic dust production. But such records based on objective measurements only span the past 100 years. Extending the comparison beyond this period, therefore, necessitates using records based on indirect evidence, such as the total amount of material ejected. In assessing the relative magnitude of the eruptions as dust producers, Lamb¹¹ includes historical accounts of (non-instrumental) observations of optical phenomena in the upper atmosphere. Lamb's volcanic dust production index (v.d.p.i.) is shown in Fig. 2a dating back to 1770. There is only one adjustment: the v.d.p.i. for the Shtyubelya Sopka eruption at 52°N in 1907 (equal to that of Katmai, 1912) has been reduced by a factor of 5 in view of radiation measurements in the 30–60° northern latitude belt¹². The open bars are mainly derived from an assumed relationship between climatic temperature anomalies and atmospheric dust veils.

In order to estimate the volcanic ion concentration in Greenland precipitation, the v.d.p.i. has to be corrected to account for the residence time of the aerosols in the atmosphere and the non-uniform distribution pattern of the fall-out. The correction has been made by accounting for (1) a temporal distribution of the fall-out corresponding to a residence time of 1 yr for aerosols in the high latitude stratosphere (2) a time lag of 1 yr for the high northern latitude fall-out of volcanic debris originating from eruptions south of 50°N, and (3) spreading of the eruption cloud on its travel to high northern latitudes by using a correction factor F on the v.d.p.i. of all eruptions. For simplicity, and with a view to fall-out data for fission products^{13,14}, supplemented by total β -activity data from the polar ice sheets^{15,16}, the F -factor is equal to 1 for latitudes of eruptions north of 50°N, 0.5 in the 20°–50°N latitude belt, 0.2 in the 20°S to 20°N belt and 0.07 for eruptions south of 20°S.

As precipitation contains a representative sample of the aerosols in the precipitating air mass, the procedure described above leads to a new index (Fig. 2b), which may be called a volcanic impurity concentration index (v.i.c.i.) for high northern latitude precipitation.

Material and techniques

Specific conductivity profiles have been measured along three ice cores recovered from North (Hans Tavsén), Mid (Crête) and South (Dye 3) Greenland (Fig. 1), all recovered under the Greenland Ice Sheet Program. The Crête σ profile is continuous through the period AD 1770–1972. The Crête and Dye 3 cores are absolute-dated¹⁶, whereas the Hans Tavsén core is dated by ice flow model considerations back to 1815 and from then on by counting annual cycles in the micro-particle concentration¹⁶.

The cores were sampled in sequence averaging four samples for each annual layer as revealed by seasonal cycles in heavy oxygen-isotope concentration¹⁶. The individual samples were cut from the cores at -12°C in a clean air bench after removal of a more



Fig. 1 The Greenland Ice Sheet with the drill sites, where the investigated ice cores were recovered: Dye 3 (elevation $e = 2,480$ m; snow accumulation rate $a = 49$ g cm^{-2} yr^{-1}); Crête ($e = 3,170$ m; $a = 25$ g cm^{-2} yr^{-1}); and Hans Tavsén ($e = 1,200$ m; $a = 17$ g cm^{-2} yr^{-1}).

than 5 mm surface layer that contains essentially all of the relevant contaminants in an undamaged core. Occasional cracks and other regions of possible deep contamination were avoided. The ice samples were melted in clean plastic beakers, and the solid micro-particle concentration including particles down to 0.05 μm radius, was measured by a light scattering method⁸ and also in many cases by the standard Coulter counting method. Often the pH was measured by standard procedures. The conductivity measurements required larger amounts of water. In order to save core material, it was necessary to dilute most of the samples up to three times with deionised, redistilled water ($\text{pH} = 5.6$, specific conductivity $\sigma = 130$ $\mu\text{S m}^{-1}$). This introduced some false noise in the low σ values, and perhaps up to a 15% increase in the σ background. The mixtures were allowed to saturate with the carbon dioxide in the ambient air at 25 °C, which changed σ by less than 10%, because both the ice¹⁷ and the dilute were near saturation beforehand. Finally, σ was determined by standard procedure.

Results and discussion

The annual mean solid micro-particle mass concentration at Crête, determined by total number and size distribution analyses, is plotted in μg per kg of ice in Fig. 2d. There is no significant correlation with the v.i.c.i. in section b, which indicates that

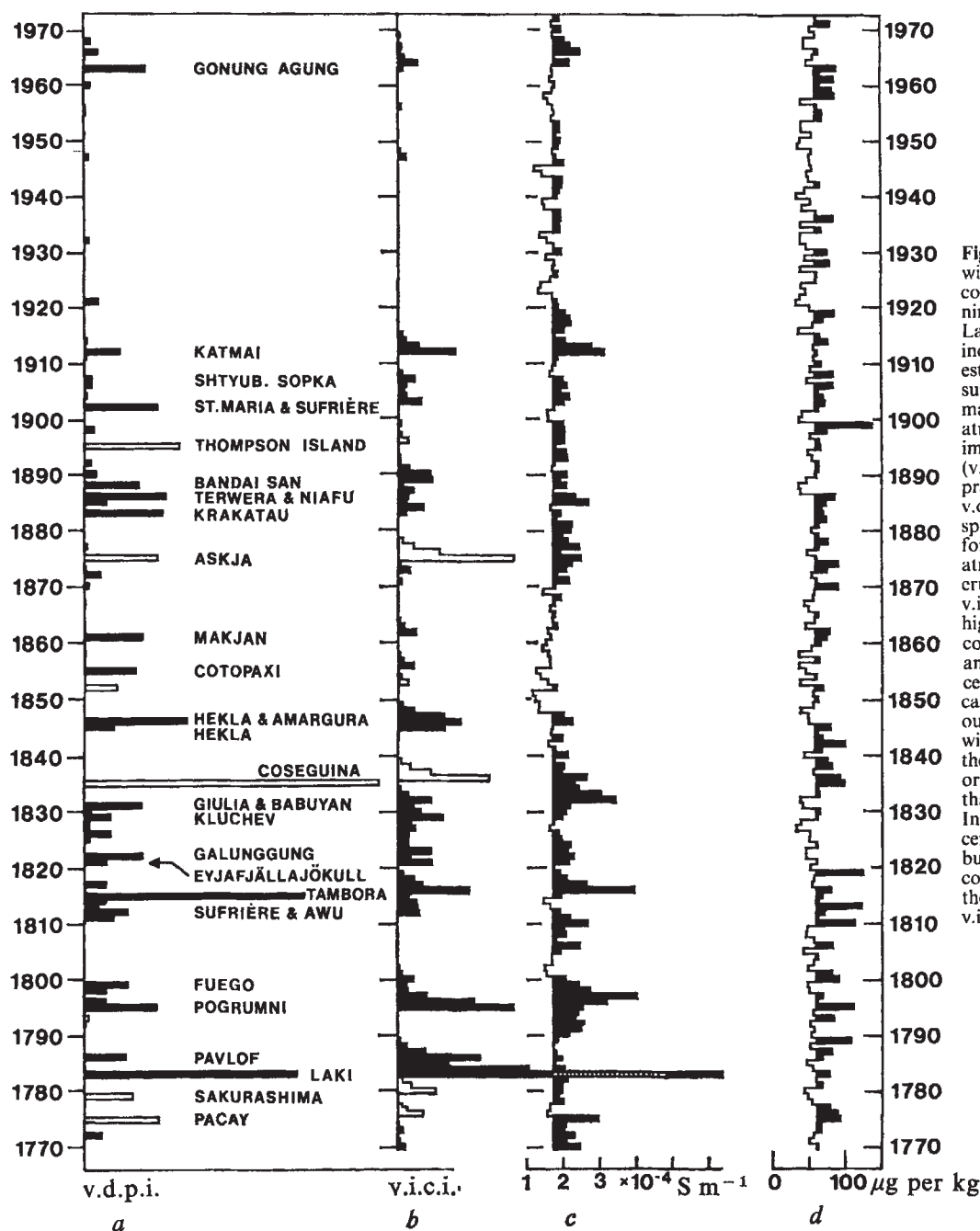


Fig. 2 Volcanic activity compared with impurity profiles along an ice core from Crête, Greenland, spanning the period 1770-1972. *a*, Lamb's volcanic dust production index (v.d.p.i.); open bars refer to estimates mainly based on an assumed relationship between climatic temperature anomalies and atmospheric dust veils. *b*, Volcanic impurity concentration index (v.i.c.i.) for high northern latitude precipitation, derived from the v.d.p.i. by correction for latitudinal spreading of the eruption cloud and for residence time of aerosols in the atmosphere. The Icelandic Laki eruption, 1783, stands out with a v.i.c.i. more than twice the second highest v.i.c.i. (1795). *c*, Specific conductivity of melted samples of annual precipitation. Values exceeding the mean in the volcanically quiet period 1920-60 are set out in black. Significant correlation with the v.i.c.i. shows that part of the soluble impurities of volcanic origin. The value for 1783 is more than twice the second highest. *d*, Insoluble micro-particle mass concentration (in μg per kg of ice). The bulk of insoluble impurities must be continental surface dust, because the curve has no correlation to the v.i.c.i., and no increasing trend in the industrial period.

essentially all of this fallout at Crête is of continental surface origin. Furthermore, the lack of significant increase in the upper part of curve *d* shows that little, if any, industrial solid dust reaches Central Greenland.

According to Fig. 2*b* the Icelandic Laki eruption in 1783 (historically the greatest lava extrusion, some 12 km^3 , on Earth) was presumably responsible for the highest fall-out of volcanic debris in Greenland in the past 200 years, which suggests the 1783 ice layer as favourable for a pilot test. Figure 3 shows σ profiles through the annual layers deposited in the period 1780-86. The shaded part of the σ s represents the contribution of H^+ , σ_{H^+} , calculated from the pH data and corrected for CO_2 induced ions. The Laki eruption is revealed in the Dye-3 and Crête cores by elevated σ and σ_{H^+} values in the layer previously absolute-dated at 1783. In the Hans Tavsén core the peak values appear in a layer dated at 1784 mainly by the less accurate ice flow model calculations. The Hans Tavsén peaks are bipartite due to a layer of refrozen meltwater that has percolated from a younger summer layer. At this station, located on a medium altitude, separate ice sheet, and only some 15 km from snow-free areas, the ice chemistry is complicated by the dust content being more than an

order of magnitude higher than at the other stations. The σ_{H^+} shows that melted Hans Tavsén precipitation has a strong acid reaction only in periods of heavy volcanic fall-out.

In Fig. 2*c* the annual mean σ values at Crête are plotted over the period 1770-1972. Those exceeding the 40 yr mean σ value ($170 \mu\text{S m}^{-1}$) for the volcanically quiet period 1920-60 are set out in black. The variability of σ in this period should be considered as noise, and deviations of the same order in the rest of the curve are therefore insignificant. Nevertheless, the small rise in σ in the late 1910s may be significant, because the HCl and HF production in 'the valley of 10,000 smokes' created by the Katmai eruption has been estimated¹⁸ at $1.4 \times 10^9 \text{ kg yr}^{-1}$ up to 1920.

Sudden rises in v.i.c.i. and σ generally occur simultaneously or with a short time lag in σ . As for eruptions south of 50°N any time lag in σ between -1 and $+1$ year may occur, in view of the general 1 yr shift introduced when transferring the Lamb's v.d.p.i. into the v.i.c.i. in Fig. 2*b*. Eruptions north of 50°N should be revealed by sudden increases in σ with time lags between zero and $+1$ yr, assuming the ice core time scale to be exact.

Although Lamb's v.d.p.i. (Fig. 2*a*) is not specifically designed to represent the volcanic production of gases and ionic com-

Table 1 Background composition of impurities in melted Greenland glacier ice

Element	Concentration ($\mu\text{mol kg}^{-1}$)	Location	Ref.
H	3.5	Crête	This work
Na	1.0 average	Dye 3, Crête	10
Si	1.4	135 km WSW of Crête	19
Al	0.3	300 km WSW of Crête	2
NH ₄	0.2	300 km WSW of Crête	2
	0.3	Crête	10
Ca	0.2	Dye 3	(Busenberg & Langway, personal communication)
SO ₄	0.8	Crête	10
		Dye 3	20
Cl	1.6	300 km WSW of Crête	2
		Dye 3	20

pounds, a statistical analysis of the v.i.c.i. and σ time series shows a highly significant correlation coefficient ($R = 0.6$; $P > 99.9\%$ for $n = 20$ degrees of freedom). Furthermore, Fourier spectral analysis shows less than 1 yr time lag for any frequency higher than 0.02 yr^{-1} , the coherence being of the order of 0.6 for the same frequencies.

If, consequently, elevated σ values are accepted as indicative of fallout of volcanic acids produced in the northern hemisphere, Fig. 2c suggests that unnoticed eruptions of considerable magnitude took place around 1810. Furthermore, the v.d.p.i. for the Askja eruption, 1875 (and Pavlof, Alaska, 1786?) may have been overestimated, as an Icelandic eruption of the estimated magnitude would probably be revealed by more than a barely significant increase in σ .

Some of the available ice sheet chemistry data are presented in Table 1. They suggest a simple explanation of the $170 \mu\text{S m}^{-1}$ σ background at Crête being due mainly to the H^+ , Na^+ , NH_4^+ , SO_4^{2-} , Cl^- and HCO_3^- . First, quite a few elements (such as, Si, Al and Mg) may be disregarded, because they are mainly present in solid micro-particles that do not contribute to σ . For example, using the Al content ($5 \mu\text{g}$ per kg ice) as an indicator of igneous rock material²¹ gives an order of $60 \mu\text{g}$ of insoluble material per kg of ice, in agreement with the overall mean value in Fig. 2d. The Ca concentration is seven times higher than in igneous rocks, but still insignificant for σ . Second, CO_3^{2-} can be disregarded, because at Crête the pH averages 5.46 in non-volcanic periods (lower in volcanic periods). The pH corresponds to a background HCO_3^- concentration [HCO_3^-] of $1.3 \mu\text{mol kg}^{-1}$, and [H^+] of $3.5 \mu\text{mol kg}^{-1}$.

The total anion concentration must of course be balanced by the total cation concentration:

$$[\text{H}^+] + [\text{Na}^+] + [\text{NH}_4^+] = 2[\text{SO}_4^{2-}] + [\text{Cl}^-] + [\text{HCO}_3^-].$$

The $1.0 \mu\text{mol kg}^{-1}$ [Na^+] originates from sea salts, which also accounts for the equivalent part of the [Cl^-]. Similarly, the $0.3 \mu\text{mol kg}^{-1}$ [NH_4^+] may be interpreted as being due to $(\text{NH}_4)_2\text{SO}_4$ of continental origin, thus accounting for $0.15 \mu\text{mol kg}^{-1}$ [SO_4^{2-}]. Subtracting these concentrations, and using the values listed in Table 1, the equation shows an essential balance between the $3.5 \mu\text{mol kg}^{-1}$ [H^+] and $0.8 - 0.15 = 0.65 \mu\text{mol kg}^{-1}$ [SO_4^{2-}] plus $0.6 \mu\text{mol kg}^{-1}$ [Cl^-] plus $1.27 \mu\text{mol kg}^{-1}$ [HCO_3^-], a total corresponding to $3.2 \mu\text{mol kg}^{-1}$ monovalent anion. Other elements may be present in minor amounts, but according to the above, H_2SO_4 contributes 33%, CO_2 -induced ions 29%, HCl 15%, NaCl 8% and $(\text{NH}_4)_2\text{SO}_4$ 3%, a total of 89% of the σ background, $170 \mu\text{S m}^{-1}$, at Crête.

Looking at the Laki event as revealed in the Dye 3 ice (Fig. 3) the σ peak value (in part of the mid 1783 layer) is $\sigma = 1,400 \mu\text{S m}^{-1}$. The pH is low, corresponding to [H^+] = $33 \mu\text{mol kg}^{-1}$, and the [SO_4^{2-}] amounts to $12 \mu\text{mol kg}^{-1}$. Hence, in addition to SO_4^{2-} , other anions (probably Cl^- , but this is not important here) contribute $33 - (2 \times 12) = 9 \mu\text{mol kg}^{-1}$. These concentrations fully account for the measured σ peak value.

The lack of a recent increasing trend of the Crête σ curve (Fig. 2c) suggests that industrial sulphates do not reach Mid Greenland

in appreciable amounts. Other authors^{20,22} have drawn the opposite conclusion from North and South Greenland data, showing sulphate concentrations in the period 1964–71 that are roughly twice the concentration in pre-industrial periods. The σ curve in Fig. 2c suggests however, that the Agung eruption 1963 may be responsible for the elevated sulphate (and chlorine) values in the late 1960s, although detailed chemical analyses of a longer and continuous part of the ice cores are needed for firm evidence.

The rise of the excess [H^+] in ice layers containing debris from a high northern latitude eruption can be used to roughly estimate the global H^+ fall-out by applying the same latitudinal distribution pattern as in the case of ^{90}Sr fall-out from high northern latitude bomb tests. In 1962, the global fall-out of ^{90}Sr (mainly from the Soviet tests in 1961) was 1.2 MCi which was 8.6×10^8 times the fall-out per km^2 at Crête (H. B. Clausen, personal communication). Applying this factor to the fall-out of volcanic H^+ per km^2 in 1783 at Crête (2.4 kg km^{-2}) gives a total fall-out of 2×10^6 tons H^+ during the Laki eruption, or $100 \times 10^9 \text{ kg}$ of mainly H_2SO_4 , as shown in Table 2. Similar estimates can be made for eruptions south of 50°N , if the F -factor is accounted for. For example, the Tambora eruption in 1815 seems to have contributed no less than $300 \times 10^9 \text{ kg}$ of acids to the stratospheric aerosol load. As to the few violent eruptions that have occurred within the period of instrumental observations, the Katmai eruption in 1912 is revealed by a change in [H^+] high enough to allow a fairly accurate estimate for comparison with independent estimates. A more detailed σ curve shows that the fall-out from the Katmai eruption lasted approximately 1.5 yr, and the total production of $\text{H}_2\text{SO}_4 + \text{HX}$ is estimated at $20 \times 10^9 \text{ kg}$. This is close to Junge's estimate⁶ of $16 \times 10^9 \text{ kg}$ total mass, suggesting that most of the Katmai aerosols were acids. But, chemical

Fig. 3 The Icelandic Laki eruption, 1783, revealed in the Dye 3, Crête and Hans Tavsén ice cores by peak values of specific conductivity in melted samples. The Hans Tavsén ice core is not dated absolutely, so the high conductivity layer may very well originate from 1783. The shaded areas show the contribution of hydrogen ions, not induced by CO_2 .

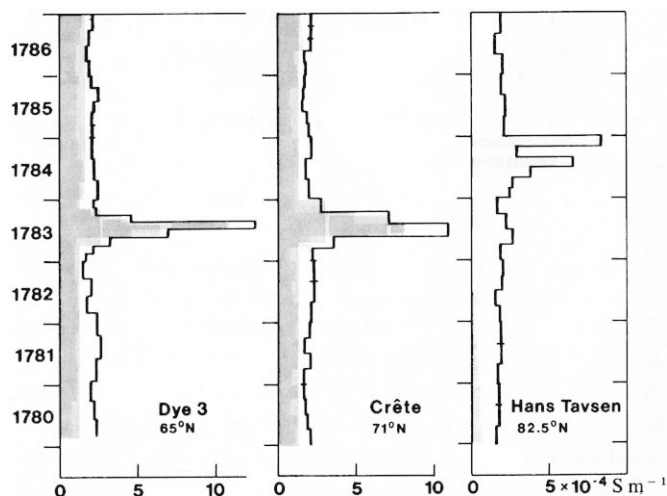


Table 2 Estimates of global fall-out of volcanic acids based on pH analyses on the Crête ice core

Eruption	Duration of deposition of debris yr	Precipitation during period of deposition $\text{kg km}^2 \times 10^8$	Average rise in (H) through deposition $\mu \text{mol kg}^{-1}$	Fall-out of volcanic H at Crête kg km^2	F	Global fall-out H^+ 10^9 kg	$\text{H}_2\text{SO}_4 + \text{HX}$ 10^9 kg	Other estimates Total mass 10^9 kg	Ref.
Laki, 1783	0.75	2	12	2.4	1	2	100		
Tambora, 1815	1	2.5	5.2	1.3	0.2	6	300		
Krakatau, 1883	2	5	(0.4)	(0.2)	0.2	(0.9)	(45)	32	(6)
Katmai, 1912	1.5	2.8	1.6	0.5	1	0.4	20	16	(6)

analyses are needed to reliably assess the magnitude of several medium sized low latitude eruptions (such as Agung, 1963) and to clarify the reasons for the instability of the background in the σ profile. Furthermore, in one case (the Aleutian eruptions 1795 and 1796), the volcanic fall-out had a pH as high as 5.53; consequently, the high σ in this period must be due to high concentrations of other components ($(\text{NH}_4)_2\text{SO}_4$, NH_4Cl ?) of relatively low equivalent conductances, which, of course, influences the estimation of the magnitude of these eruptions.

The possible influence of atmospheric turbidity on climate is complicated, because aerosols contribute to the albedo as well as the absorption of radiation. The net-effect upon global temperatures is poorly known. For comparison with climatic records, the σ profile is used to design an objective volcanic aerosol production index (by a procedure opposite to that used to design the v.i.c.i.) and turn it into an index of possible influence of volcanic aerosols upon northern hemisphere temperatures, with due account for the latitudes of the eruptions and for the residence time of the aerosols in the atmosphere. This index has no significant correlation to annual mean temperatures in England²³, even with time lags up to 10 yr ($R \sim -0.06$ to -0.19 ; $P \sim 30$ to 70% ; $n = 20$), which cannot be due solely to the index being imperfect. The lack of correlation confirms the result of a similar attempt²⁴ using Lamb's dust veil index. When using 10-yr averages, one finds a high but insignificant correlation ($R = -0.55$, $P = 93\%$, $n = 8$). This procedure is not advisable, because it may mask important time lags. For example, the cooling in the 1960s began several years before the Agung eruption 1963. Other authors²⁵ have claimed that volcanic aerosols have made important contributions to climatic changes in the past, but they only considered details of the 1884–1970 period that seems to include too few great volcanic events to justify a statistically significant statement.

In conclusion, specific conductivity profiles reveal violent volcanism in the past. Elevated conductivities, due to fall-out of acids, usually allow the contribution of the eruptions to the high altitude aerosol budget to be estimated if the locations of the eruptions are known. If they are unknown, comparison of conductivity profiles along ice cores recovered at different latitudes in Greenland and Antarctica may lead to estimates of the

volcanic activity beyond the range of historical records, at least 10,000 years ago, and particularly when combined with chemical analyses. Ice from the Wisconsin glaciation was deposited in a different climatic regime and contains high and strongly variable amounts of chemical elements⁴, which may complicate the interpretation. One way of using long volcanic activity records would be to check if they have any relation to climatic changes as revealed by stable isotope profiles along the same cores. Another aspect concerns the high concentrations of hydrogen ions in layers deposited in periods of high volcanic activity, which suggests anomalous dielectric properties of such ice. Series of layers containing high concentrations of volcanic impurities is, therefore, a possible explanation of the internal reflection layers encountered in radio-echo sounding²⁶.

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Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone

Peter H. Seeburg, John Shine, Joseph A. Martial, John D. Baxter & Howard M. Goodman

Department of Biochemistry and Biophysics and Metabolic Research Unit, University of California, San Francisco, California 94143

The primary structure of DNA containing the sequence for rat pituitary growth hormone mRNA has been determined. DNA was obtained by reverse transcription of polyadenylated RNA from cultured pituitary cells and from recombinant bacterial plasmids. The amino acid sequences for rat growth hormone and its precursor form have been deduced from the determined nucleotide sequences.

SOMATOTROPIN (growth hormone, GH) is essential for linear growth in man and other vertebrates. In childhood, deficiency of this peptide hormone results in dwarfism whereas overproduction causes gigantism. In the adult, pathological overproduction of growth hormone can lead to acromegalic deformations^{1,2}. Somatotropin is separable in activity and substance from the related pituitary hormone prolactin^{3,4}. Comparisons of primary structures have revealed that these hormones, together with the placental hormone chorionic somatomammotropin form a group of sequence-related peptides of molecular weights (MW) around

22,000, with a suggested common evolutionary origin⁵⁻⁷. The three hormones also share certain features in their biological actions⁸.

The genes for somatotropin and related hormones thus provide an excellent system for studying structure, evolution and regulation of a set of similar DNA sequences whose expression is ordinarily confined to separate tissues (pituitary and placenta) and is elicited by different physiological stimuli. While information on the structural gene for human chorionic somatomammotropin has very recently been obtained by us^{9,10}, no such information has been available for growth hormone.

We report here the *in vitro* synthesis, determination of the nucleotide sequence, and the amplification in bacteria of structural gene sequences for growth hormone starting with mRNA from cultured rat pituitary cells¹¹. These cells can be hormonally induced to produce and secrete somatotropin^{12,13}. In analogy to other secreted proteins, synthesis of this peptide occurs at the rough endoplasmic reticulum as a precursor form¹⁴⁻¹⁶. The N-terminal portion (signal peptide) of the precursor is thought to be involved in the attachment of ribosomes to membranes, a process essential for hormone maturation and secretion (for model on mechanism see ref. 17). Accordingly, the primary translation product of rat growth hormone mRNA is a peptide larger (by about 3,000 MW units, see Fig. 1) than the mature hormone^{13,16}. As the primary structure of rat somatotropin is only partially known¹⁸, the nucleotide sequence determined for the mRNA allows us to predict the complete amino acid sequence of the hormone including the previously unknown sequence of the signal peptide.

Detection and sequence determination of DNA complementary to growth hormone mRNA

Certain tissues in which the mRNA for a specialised function represents a large proportion of the polyadenylated RNA (for example, globin RNA in reticulocytes¹⁹, ovalbumin RNA in induced oviducts²⁰, insulin RNA in the islets of Langerhans^{21,22}) allow for their convenient detection and characterisation of such RNAs or their cDNAs. In cultured rat pituitary cells (strain GC), however, when grown in normal conditions, growth hormone mRNA represents only a small percentage (1-3%) of the total poly A containing RNA¹³. Therefore, the detection and isolation of nucleic acid sequences specific for rat growth hormone were approached by a strategy previously used to obtain sequences from polyadenylated RNAs that constitute 2% or more of the cellular messengers⁹. This strategy involves restriction endonucleolytic cleavage of complex cDNA mixtures (single or double stranded) prepared by reverse transcription of polyadenylated cellular RNAs, followed by gel electrophoretic separation of the resultant cDNA fragments. In applying this approach to the detection of growth hormone cDNA we were aided by the fact that in cultured rat pituitary cells growth hormone mRNA levels can be raised above that of other cellular mRNA species by the synergistic action of thyroid hormones and glucocorticoids^{13,23}. Further enrichment of growth hormone mRNA was achieved by isolating poly A-containing RNA from cytoplasmic membranes²⁴ where growth hormone is synthesised. In the final RNA template used for cDNA synthesis, growth hormone mRNA was therefore the most abundant species representing about 10% of the RNA (see Fig. 1).

Analysis by gel electrophoresis of this cDNA after cleavage with endonucleases *Hae*III and *Hha*I is shown in Fig. 2. Each enzyme generates several abundant DNA fragments which can be seen as prominent bands above the polydisperse background of the restricted cDNA. Whereas *Hae*III yields a complex fragment pattern (Fig. 2b), cleavage with *Hha*I produces a simpler pattern characterised by two major DNA fragments, approximately 320 (fragment A) and 240 (fragment B) nucleotides long (Fig. 2c). The prominence of fragments A and B suggests that they may be derived from growth hormone mRNA.

For further characterisation, these fragments were isolated from a polyacrylamide gel on which approximately 1 µg of *Hha*I-

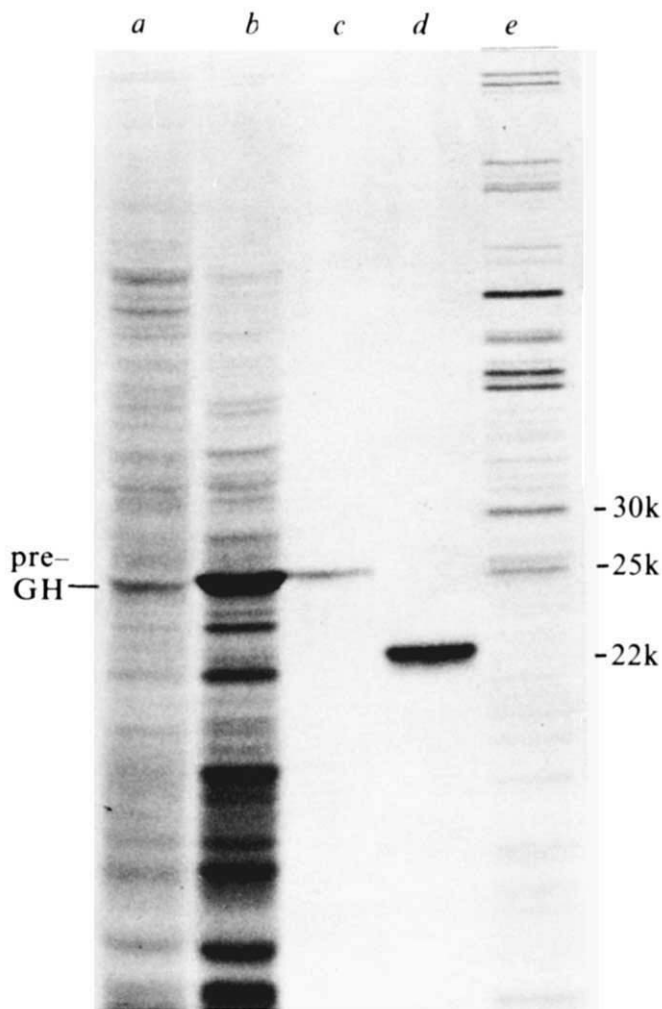


Fig. 1 Translation products of rat growth hormone mRNA. Polyadenylated RNA was isolated from the cytoplasmic membrane fraction of cultured rat pituitary cells (strain GC) as described elsewhere^{13,24}. Usually, 30 µg of poly A-containing RNA were obtained from 5×10^8 cells grown in suspension culture and induced for GH production by including 1 µM dexamethasone and 10 nM L-triiodothyronine in the medium for 4 d before cell collection. Polyadenylated RNA was also isolated from cells grown for 4 d in the absence of dexamethasone and triiodothyronine²⁵. Aliquots of these RNA preparations (500 ng) were translated in a cell-free translation system derived from wheat germ, and the products immunoprecipitated using rhesus monkey antiserum to rat GH as previously described¹³. To obtain labelled rat growth hormone, induced cells (about 10^6 cells in 1 ml) were pelleted, resuspended in 0.2 ml of serum- and methionine-free medium containing bovine serum albumin ($0.5 \mu\text{g ml}^{-1}$), and labelling was carried out with ^{35}S -methionine ($50 \mu\text{Ci}$, NEN) for 4 h at 37°C . Cells were pelleted and labelled rat GH released into the medium was immune-precipitated as described above. Protein products of cell-free translations and immunoprecipitations were separated in 12.5% polyacrylamide slab gels ($14 \times 14 \times 0.2 \text{ cm}$) in the presence of sodium dodecylsulphate⁵⁶ at 20 mA per gel for 4 h. Gels were soaked in 50% trichloroacetic acid, washed in 7% acetic acid, dried on paper (Whatman 3MM), and exposed to X-ray film (Kodak NS2T). The autoradiogram shown contains a, translation products of poly A 'membrane' RNA from cells grown in the absence of dexamethasone and thyroid hormone; b, the same as (a) except that the RNA was isolated from hormone-induced cells; c, immune-precipitate of (b); d, immune-precipitate of secreted rat GH; e, ^{14}C -labelled proteins from bacteriophage T4-infected *E. coli* cells⁵⁷ as size markers.

restricted cDNA had been separated. Each fragment was further cleaved by *Hae*III (see Fig. 2c) and the products ($A_1 \sim 180$, $A_2 \sim 75$, $A_3 \sim 65$, $B_1 \sim 140$, $B_2 \sim 100$ nucleotides) enzymatically labelled with ^{32}P at their 5' termini. Following gel electrophoretic separation, the five labelled DNA fragments were processed for nucleotide sequence determination according to

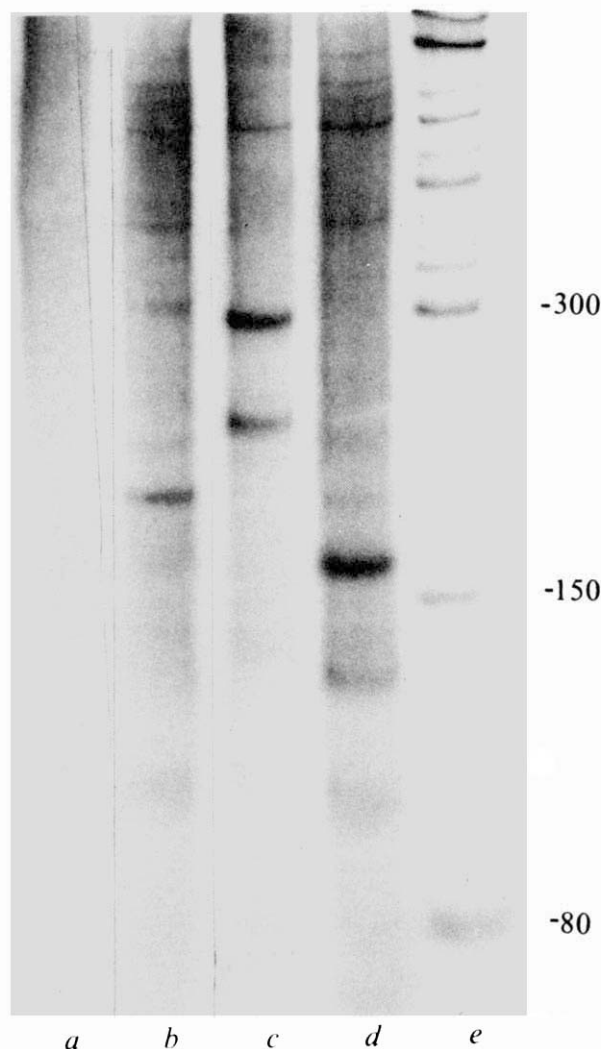


Fig. 2 Restriction endonuclease cleavage of single-stranded cDNA to rat pituitary cell RNA. Aliquots (300 ng) of the polyadenylated RNA prepared as described in Fig. 1, were transcribed into cDNA in 5- μ l reactions containing 50 ng oligo-dT₁₂₋₁₈ (Collaborative Research), 50 mM Tris-HCl, pH 8.1, 20 mM KCl, 7 mM MgCl₂, 0.1 mM EDTA, 10 mM β -mercaptoethanol, 500 μ M each of dGTP, dCTP, dTTP, 30 μ M α -³²PdATP (specific activity 5×10^4 c.p.m. pmol⁻¹), and 5 U reverse transcriptase. DNA synthesis proceeded for 7 min at 42 °C and incorporation of ³²P-dAMP into DNA was approximately 2×10^6 c.p.m. Reaction mixtures were diluted with 20 μ l of H₂O, heated to 95 °C for 2 min, quick-cooled, adjusted to 7 mM MgCl₂, and split into 5 μ l aliquots for restriction endonuclease cleavage of cDNA. Digestion with *Hae*III (2 U), or *Hha*I (2 U), or both enzymes⁹ was for 60 min at 37 °C. Mixtures were made 10 mM in EDTA, 10% in sucrose, 0.2% in sarcosyl, 0.05% in bromophenol blue (final volume 10 μ l), heated to 95 °C for 2 min, quick-cooled, loaded on to 4.5% polyacrylamide slab gels (14 $\times$ 14 $\times$ 0.2 cm) in TBE buffer⁵⁸, and electrophoresed at 150 V for 2 h. Gels were dried and exposed to X-ray film (Kodak NS2T). The autoradiogram shown (obtained after 4 h of exposure) contains a, uncleaved cDNA; b, *Hae*III-cleaved cDNA; c, *Hha*I-cleaved cDNA; d, cDNA cleaved with *Hae*III and *Hha*I; e, *Hae*III-cleaved ³²P-labelled phage M13 single-stranded DNA⁵⁹ as molecular weight marker. Numbers indicate approximate sizes in nucleotides.

Maxam and Gilbert²⁵. Typical examples of such determinations are shown in Fig. 3, and the nucleotide sequences are compiled in Fig. 7.

To identify their origin, the cDNA sequences were converted into their antiparallel complementary RNA sequences and the coding potential in the three possible phases was compared with the known amino acid sequences of several vertebrate GHs²⁶, including parts of rat growth hormone determined by Wallis and Davies¹⁸. This comparison showed that cDNA fragments A and B clearly originate from rat growth hormone mRNA. This result emphasises the experimental advantage of analysing DNA reverse transcribed from complex cellular RNA mixtures for

obtaining primary structural information on abundant mRNA species.

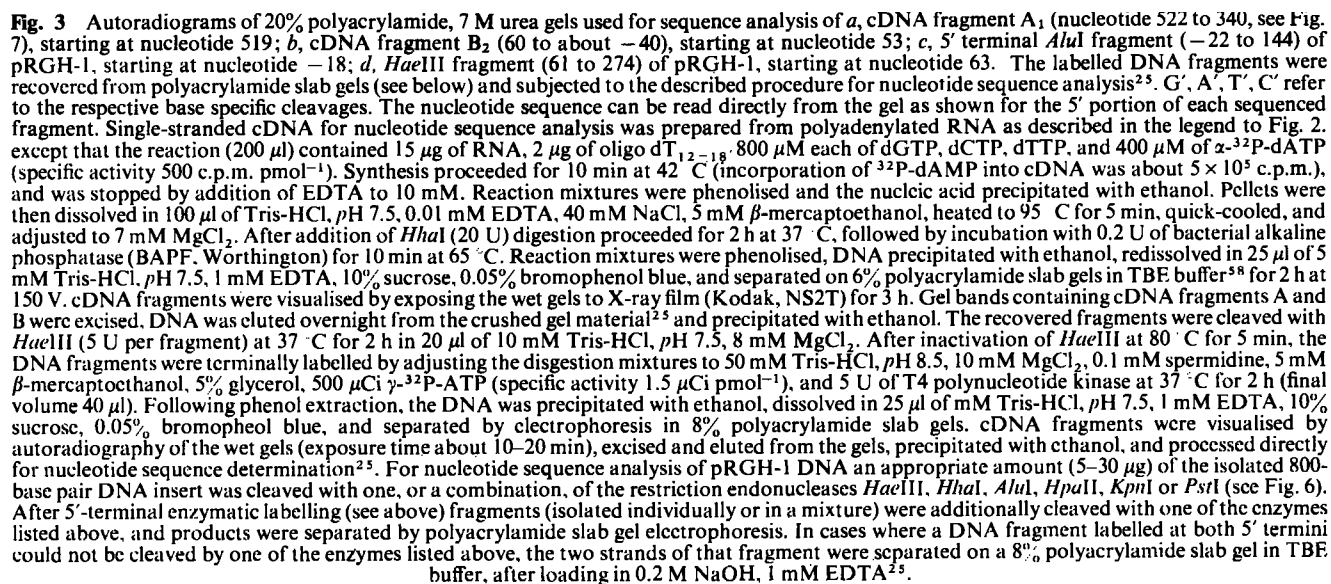
Fragments A and B are complementary to a contiguous segment of the mRNA, extending from the codon for amino acid 149 to approximately 40 nucleotides to the 5' side of the AUG initiation codon for pre-growth hormone (see Fig. 8). Since the distance of some 40 nucleotides from the end of fragment B to the initiation codon for pre-growth hormone (see Fig. 8). As the region in other eukaryotic mRNAs^{27,28}, it is possible that the 3' terminus of fragment B corresponds to the 5' terminus of the mRNA, and is not generated by *Hha*I cleavage (see also later).

Molecular cloning of growth hormone structural gene sequences

To obtain a convenient source of growth hormone DNA for further analyses, we constructed bacterial plasmids containing the structural gene for rat growth hormone. The cDNA fragments A and B served as excellent markers for monitoring construction of the correct plasmids. The starting material for the *in vitro* synthesis and subsequent cloning of the structural gene sequences was a cDNA preparation comparable in amount and purity to the one used for sequencing fragments A and B. By using its self-priming properties, this cDNA was enzymatically converted to its duplex form characterised by a hair-pin structure on one end and a poly(dA-dT) tract on the other²⁹. S1 nuclease was used to open the terminal hair-pin loop and to degrade any single-stranded DNA. Gel analysis of the DNA material before and after nuclease treatment (Fig. 4b, c) demonstrates the presence of a double-stranded DNA of approximately 800 base pairs faintly detectable above the disperse background of other DNA. An aliquot of this DNA species was purified by gel electrophoresis and cleaved with *Hha*I. Two prominent fragments corresponding in size to DNA fragments A and B were among the major cleavage products (see Fig. 4i). As fragment B is complementary to the 5' portion of growth hormone mRNA (see above), this finding indicated that the 800-base pair DNA contained primarily full-length copies of the messenger.

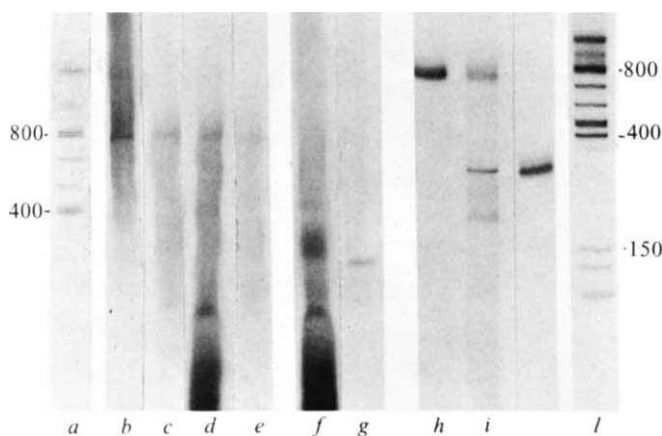
To allow for reversible insertion into a bacterial cloning vehicle, the double-stranded cDNA was given cohesive ends by enzymatic blunt-end ligation³⁰ to chemically-synthesised restriction site linkers, and subsequent cleavage with the appropriate restriction endonuclease^{31,32}. The linkers had the self-complementary sequence 5'-CCAAGCTTGG-3', containing the recognition site for *Hind*III. This site is not present in the 800-base pair DNA (data not shown) and occurs once in the bacterial plasmid pBR322 chosen for cDNA insertion (see below). Preceding ligation to linkers, the S1 nuclease-treated double-stranded cDNAs were 'blunt-ended' using *Escherichia coli* DNA polymerase I in the presence of deoxynucleoside triphosphates³³ (see legend to Fig. 4). This step ensured a high participation of the cDNA molecules in the ligation reaction, as shown by the blurred appearance due to multiple linker additions, of both the 800-base pair DNA and DNA fragments A and B (see Fig. 4d, f). Fragments A and B had been obtained by *Hha*I-cleavage of the double-stranded cDNA before S1-treatment and their 3'-CG ends removed by *E. coli* DNA polymerase I before linker attachment. The ligation products were cleaved with *Hind*III (*Hsu*I endonuclease was used interchangeably with *Hind*III) to generate DNA molecules with cohesive termini and to digest self-ligated linkers (Fig. 4e, g). The 800-base pair DNA and DNA fragments A and B were purified on a polyacrylamide gel (Fig. 4h, k) to reduce the level of DNA sequences unrelated to growth hormone that would enter the bacterial cells.

The EK-2 vector pBR 322³⁴, a relaxed replicating plasmid of MW 2.6×10^6 containing a single *Hind*III cleavage site was used as the cloning vehicle for growth hormone cDNA. Before insertion of the cDNA, the *Hind*III-cleaved linear plasmid was treated with bacterial alkaline phosphatase to remove the 5'-terminal phosphate groups. This step permits the selective construction of recombinant plasmids by preventing closure of the plasmid at the original cleavage site. Infectious circular plasmid forms will only be generated by bridging and closing the plasmid



Following ligation of the gel-isolated 800-base pair DNA to phosphatase-treated linear pBR322 plasmid the reaction mixture

was used to transform the bacterial EK-2 host *E. coli* X1776. (In a P3 facility to NIH guidelines) pBR322 confers host resistance to the antibiotics ampicillin and tetracycline, and DNA insertions into the *Hind*III site have been found to reduce or abolish



(see above). Fractions containing DNA were pooled and the nucleic acid precipitated with ethanol. The precipitate was redissolved in 100 μ l of 5 mM Tris-HCl, pH 7.5, 0.05 mM EDTA, and split into 50 μ l aliquots. One aliquot was adjusted to 300 mM NaCl, 30 mM Na-acetate, pH 4.5, 3 mM ZnCl₂, and incubated with 5 U of S1 nuclease (prepared according to Vogt⁶⁰) at 37 °C for 10 min followed by phenol extraction in the presence of 5 mM EDTA and precipitation of the DNA with ethanol. The second aliquot was adjusted to 10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 8 mM MgCl₂, 5 mM β -mercaptoethanol, and the DNA was cleaved with 5 U of *Hha*I for 2 h at 37 °C followed by phenol extraction and precipitation of the DNA fragments with ethanol. For ligation to the *Hind*III linkers, DNA precipitates from both aliquots were separately dissolved in 25 μ l of 60 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 10 mM β -mercaptoethanol, 1 mM ATP, and 200 μ M each of dATP, dTTP, dGTP, dCTP. They were incubated with 1 U (1 μ l) of *E. coli* DNA polymerase I at 10 °C for 10 min to exonucleolytically remove any 3' protruding ends and to 'fill in' any 5' protruding ends³³. Incubation was followed by addition of 50 pmol of 5' ³²P-labelled *Hind*III linker (10⁵ c.p.m. pmol⁻¹), in 15 μ l of 60 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 10 mM β -mercaptoethanol, 1 mM ATP, and bacteriophage T4 DNA ligase (to 20 μ g ml⁻¹)⁶¹. Ligation was for 60 min at 20 °C. Reaction mixtures were diluted with 100 μ l of 7 mM MgCl₂, 50 mM NaCl, heated at 80 °C for 5 min, cooled and DNA digested with 15 U of *Hsu*I at 37 °C for 4 h. The reaction mixtures were phenolised, DNA was precipitated with ethanol, redissolved in 25 μ l of 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% sucrose, 0.05% bromophenol blue, and separated on 4.5% polyacrylamide slab gel at 150 V for 2 h. The 800-base pair DNA and the double-stranded rat GH DNA fragments A and B were visualised by autoradiography of the wet gel and electrophoretically eluted from the isolated gel pieces. Lanes a and i, *Hpa*II-cleaved ³²P labelled phage M13 RF DNA⁶² as molecular weight marker (numbers indicate approximate sizes in base pairs); b, DNA product after second strand synthesis; c, S1 nuclease digest of the sample shown in b; d, *E. coli* DNA polymerase I treatment and ligation of *Hind*III linkers to the sample shown in c; e, digestion of sample d with *Hsu*I; f, double-stranded cDNA cleaved with *Hha*I, blunt-ended with *E. coli* DNA polymerase I, and ligated to *Hind*III linkers; g, *Hsu*I cleavage of the sample shown in f; h 800-base pair DNA with cohesive *Hind*III termini after preparative gel elution; i, *Hha*I digestion of the gel-eluted 800-base pair DNA; k, double-stranded DNA fragment A with cohesive *Hind*III termini after preparative gel elution.

Fig. 4 Preparation of double-stranded cDNA from rat growth hormone mRNA. Polyadenylated pituitary cell RNA (15 μ g) was reverse-transcribed into cDNA as detailed in Fig. 3. Synthesis was stopped by addition of EDTA to 10 mM, the reaction mixture phenolised and nucleic acid precipitated with ethanol. The precipitate was redissolved in 75 μ l of 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, fractionated on a Sephadex G-50 column (0.6 $\times$ 10 cm) in the same buffer, and fractions containing the cDNA were pooled. After precipitation with ethanol nucleic acid was redissolved in 150 μ l of 0.1 N NaOH, 1 mM EDTA, and the RNA hydrolysed at 70 °C for 20 min. After neutralisation and precipitation with ethanol, the single-stranded cDNA was converted to its duplex form in a 50- μ l reaction containing 50 mM Tris-HCl, pH 8.1, 0.1 mM EDTA, 20 mM KCl, 7 mM MgCl₂, 10 mM β -mercaptoethanol, 400 μ M each of dGTP, dTTP, dCTP, and 100 μ M of α -³²P dATP (specific activity about 10⁴ c.p.m. pmol⁻¹). The reaction mixture was heated to 90 °C for 2 min, quick-cooled, and synthesis proceeded for 2 h at 40 °C (incorporation of ³²P-dAMP into DNA was approximately 3 $\times$ 10⁶ c.p.m.) followed by addition of 2 μ g of *E. coli* tRNA, phenol extraction, and fractionation of the aqueous phase by gel filtration

tetracycline resistance³⁴. Recombinants were therefore selected by growth on nutrient plates containing ampicillin, and by their inability to grow on high levels (20 μ g ml⁻¹) of tetracycline. Ten colonies were obtained which all carried plasmid with an insert of approximately 800 base pairs that was released by *Hind*III cleavage. Consistent with the purity of the cDNA material inserted into plasmid (30–50% growth hormone DNA, see Fig. 4i), 4 of the 10 inserts were growth hormone DNA as judged by their restriction endonuclease patterns (Figs 5 and 6), and shown by direct sequencing in one case (see below).

Growth hormone DNA fragment A was successfully cloned in the same manner: 7 out of 8 clones contained plasmid with the correct DNA insertion as seen by *Hae*III and *Kpn*I digestion (see Fig. 6). Nucleotide sequence analysis of one of these inserts showed that the 3' protruding –CG termini in the double-stranded cDNA fragment A had been removed correctly by *E. coli* DNA polymerase I (see Fig. 7) demonstrating the reliability of this method for obtaining defined blunt-ended DNA molecules from those with frayed ends. No clones were obtained for DNA fragment B. This could reflect the finding (see below) that the cloned 800-base pair DNA does not contain the presumptive *Hha*I-site which was thought to terminate the single-stranded cDNA fragment B. The double-stranded form of this fragment probably retained its terminal hairpin after *Hha*I cleavage, thereby preventing the addition of linkers to this end of the molecule and its subsequent insertion into the cloning vehicle.

Sequence determination of the cloned DNA

The 800-base pair growth hormone DNA was isolated in preparative amounts from one of the recombinant clones (pRGH-1) and its nucleotide sequence determined by the method of Maxam and Gilbert²⁵ (see Fig. 3b). The entire sequence of this DNA is compiled in Fig. 7. A large part of it has been confirmed by determining the nucleotide order in both DNA strands. The sequence is identical to that determined for cDNA fragments A and B which demonstrates the fidelity of amplifying eukaryotic

structural gene sequences in bacteria, and stresses the advantage of cDNA sequencing to probe such fidelity.

The cloned 800-base pair DNA specifies the entire coding sequence for the precursor form of rat growth hormone. In addition, it contains all of the 3', and part of the 5' untranslated region of growth hormone mRNA. Failure to obtain the complete 5'-terminal sequence of a mRNA in cloned double-stranded cDNA (synthesised *in vitro* by self-priming of single-stranded cDNA and S1 nuclease treatment) has also been observed in the few other instances reported^{22,35}.

Primary structure of rat growth hormone mRNA

The DNA sequence determined from cDNA and cloned material allows us to derive the primary structure of rat growth hormone mRNA as shown in Fig. 8. The size of this RNA (excluding the poly A) is estimated to be 800 nucleotides, assuming that the 5' end corresponds approximately to the 3' end of cDNA fragment B. This estimate agrees well with previous values obtained from velocity sedimentation¹³ and gel electrophoresis³⁶ of rat growth hormone mRNA.

Only preliminary conclusions can be drawn about the 5'-untranslated part of growth hormone RNA since the full sequence has not yet been established. At least 20 nucleotides are known to be missing from pRGH-1, but a tentative sequence is available from cDNA fragment B (Fig. 7).

No obvious features characterise the established 5' portion of the mRNA sequence and no apparent homology to known 5' untranslated regions of other eukaryotic messengers is found. But, there is a potential complementarity with the 3' terminal sequence (5'-GAUCAUUA-3') of eukaryotic 18S ribosomal RNA involving nucleotides –15 to –12 in the messenger. An interaction between the 5' end of mRNA and the 3' end of ribosomal RNA has been demonstrated in the initiation of translation for prokaryotic mRNAs^{37,38}, and by analogy has been suggested to occur in eukaryotes³⁹. The linear distance from this site to the initiation codon AUG is comparable with that in rabbit β -globin RNA^{27,40} and rous sarcoma viral RNA²⁸ where

such a complementarity has also been observed.

It may be of interest that the region surrounding the AUG initiation codon can be folded into a secondary structure with the AUG being part of a hairpin loop (residues -4 to 3) and the base-paired stem interrupted by non-complementary sequences (residues -17 to -11, and 11 to 20) containing the region complementary to the 3' terminus of 18S ribosomal RNA. Such a structure (T_m^{41} approximately 50 °C) could form through interaction with proteins involved in the initiation of protein synthesis.

The 3' untranslated region of rat GH mRNA contains 105 nucleotides, excluding the poly A. Whereas the distal portion of this region is rich (73%) in A + U, 30 of the first 64 nucleotides following the UAG termination codon are C's, present mainly in stretches of 4, 5, or 6 residues. Two of these C tracts are part of a sequence of 16 pyrimidines (residues 676-693) interrupted around the centre by two purines. Such highly asymmetric distribution of purines and pyrimidines is known to influence the secondary structure of the corresponding DNA double helix⁴² and may be of significance concerning structural requirements for transcriptional regulation. It is interesting to note that the CCACCCCU sequence (nucleotides 709-716) found at the end of the C-rich region is also present in rat insulin mRNA²², and slightly modified in human α -globin⁴³ and rabbit β -globin RNA⁴⁴.

Several regions with symmetry elements occur throughout the 3'-terminal sequence of GH mRNA. Of note are four regions of a palindromic nature the first two of which (following the termination codon) overlap in sequence comprising 12 and 20 nucleotides and are centred around positions 656 and 667 respectively. The third region surrounds the GUUA sequence (residues 683-686) with two C tracts, and is reminiscent of the extended palindrome found in an equivalent position in HCS mRNA^{9,10}. The fourth palindrome (nucleotides 728-740) is solely composed of A and U residues and contains the AAUAAA stretch found in all other eukaryotic mRNAs sequenced to date⁴⁵. Between this sequence and the poly A tract a self-complementary sequence occurs (nucleotides 739-750) which would form a region of twofold symmetry in the corresponding double-stranded DNA. The functions of these symmetry elements and sequence features are unknown. These sequences may be of regulatory nature providing sites of interaction with proteins involved in the transcription of the growth hormone gene, and in the processing, packaging, and translation of growth hormone mRNA.

The poly A tract characteristic of most eukaryotic mRNAs⁴⁶ starts with nucleotide 754. As determined from the cloned DNA sequence this tract is interrupted after 10 nucleotides by a C residue. This finding probably reflects the slippage of the extended oligo-dT primer in the initial stages of cDNA synthesis. But, the existence of occasional substitutions in poly A tracts of mRNAs cannot be excluded.

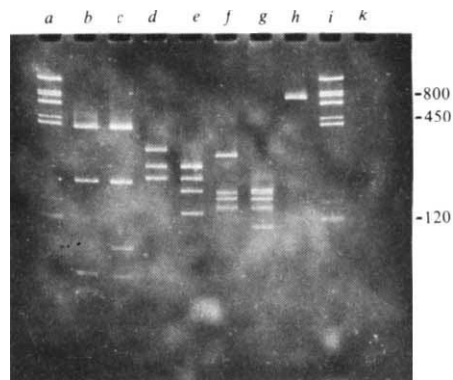
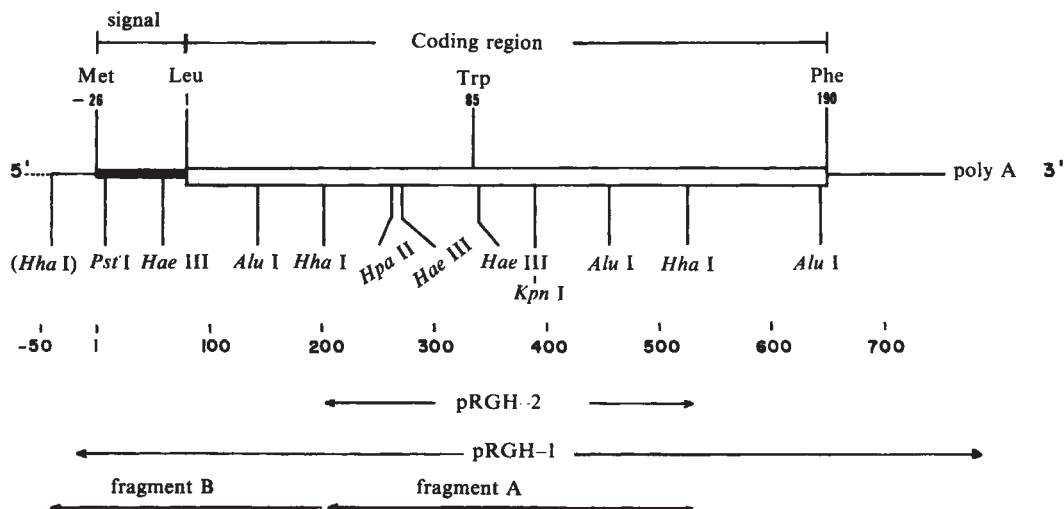


Fig. 5 Restriction endonuclease cleavage pattern of cloned 800-base pair rat growth hormone DNA. The 800-base pair DNA and DNA fragments A and B, purified by gel electrophoresis, were ligated to the EK-2 vector pBR 322³⁴ in 50- μ l reactions containing 60 mM Tris-HCl, pH 8, 10 mM β -mercaptoethanol, 8 mM MgCl₂, between 10 and 50 ng (as judged from their specific activities) of the purified DNAs, and approximately 500 ng of *Hind*III-cleaved 5'-dephosphorylated plasmid DNA. Ligations were started by addition of T4 DNA ligase (to 5 μ g ml⁻¹)⁶¹, allowed to proceed at 15 °C for 1 h, and mixtures diluted to 0.25 ml with 120 mM NaCl, 1 mM EDTA. The NIH-certified EK-2 host *E. coli* X1776 constructed by R. Curtiss III was grown in 150 ml of nutrient broth supplemented with DL- α -diaminopimelic acid (DAP, 100 μ g ml⁻¹) and thymine (40 μ g ml⁻¹) to an A_{650} of 0.2. After centrifugation the cells were washed in 60 ml of 10 mM NaCl, pelleted, resuspended in 60 ml of transformation buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 75 mM CaCl₂), kept on ice for 15 min, pelleted, and resuspended in 1.5 ml of the same buffer. The following transformation, cell growth, and the isolation of cloned, recombinant plasmid DNA were performed in a certified P3 laboratory. After adding 0.5 ml of CaCl₂-treated cells to the diluted ligation reactions the transformation mixtures (0.75 ml) were kept on ice for 15 min, at 25 °C for 4 min, and again on ice for 30 min. They were then plated directly on to nutrient plates (about 0.2 ml per plate) containing ampicillin (20 μ g ml⁻¹), DAP (100 μ g ml⁻¹), and thymine (40 μ g ml⁻¹) and the plates incubated for 24 h at 37 °C. In these conditions about 20 transformants were usually obtained from each ng of supercoiled pBR 322 plasmid. In the experiments described here transformation with the 800-base pair DNA yielded 11 colonies, one of which grew on tetracycline (20 μ g ml⁻¹) and carried plasmid without insert. cDNA fragment preparations A and B gave 8 and 5 colonies, respectively. Analysis of the isolated plasmids by restriction endonuclease cleavage and 6% polyacrylamide slab gel electrophoresis of the cleavage products (which were then visualised under ultraviolet light after staining with ethidium bromide⁶⁶) showed that 4 colonies contained 800-base pair GH DNA, 7 colonies contained GH DNA fragment A and none was found to contain GH fragment B. Shown are digests of pRGH-1 insert (800-base pair rat GH DNA) with the restriction endonucleases: *Hae*III and *Pst*I, (b); *Hae*III, (c); *Hha*I, (d); *Hha*I and *Kpn*I (e); *Alu*I (f); *Alu*I and *Hpa*II (g); no enzyme (h). Lanes (a) and (i) contain *Hpa*II-cleaved phage fd-RF DNA⁶² as molecular weight marker. Numbers indicate approximate sizes in base pairs.

Fig. 6 Physical map of rat growth hormone mRNA from the poly A to the 5' end. The black and white boxes span the coding region and represent the signal region and the region coding for the mature hormone respectively. Characteristic amino acids and their respective positions are shown above the boxed areas for orientation. The locations of the cleavage sites in the corresponding DNA for restriction endonucleases *Alu*I, *Hae*III, *Hha*I, *Hpa*II, *Kpn*I, and *Pst*I were determined from growth hormone cDNA and from cloned growth hormone DNA. It is uncertain whether the left-most *Hha*I site exists or whether this site represents the 5' end of growth hormone mRNA. The segments comprised by cDNA fragments A and B, and by the cloned fragments present in pRGH-1 and pRGH-2 are indicated. Nucleotide numbers are given below the symbols for the endonucleases.



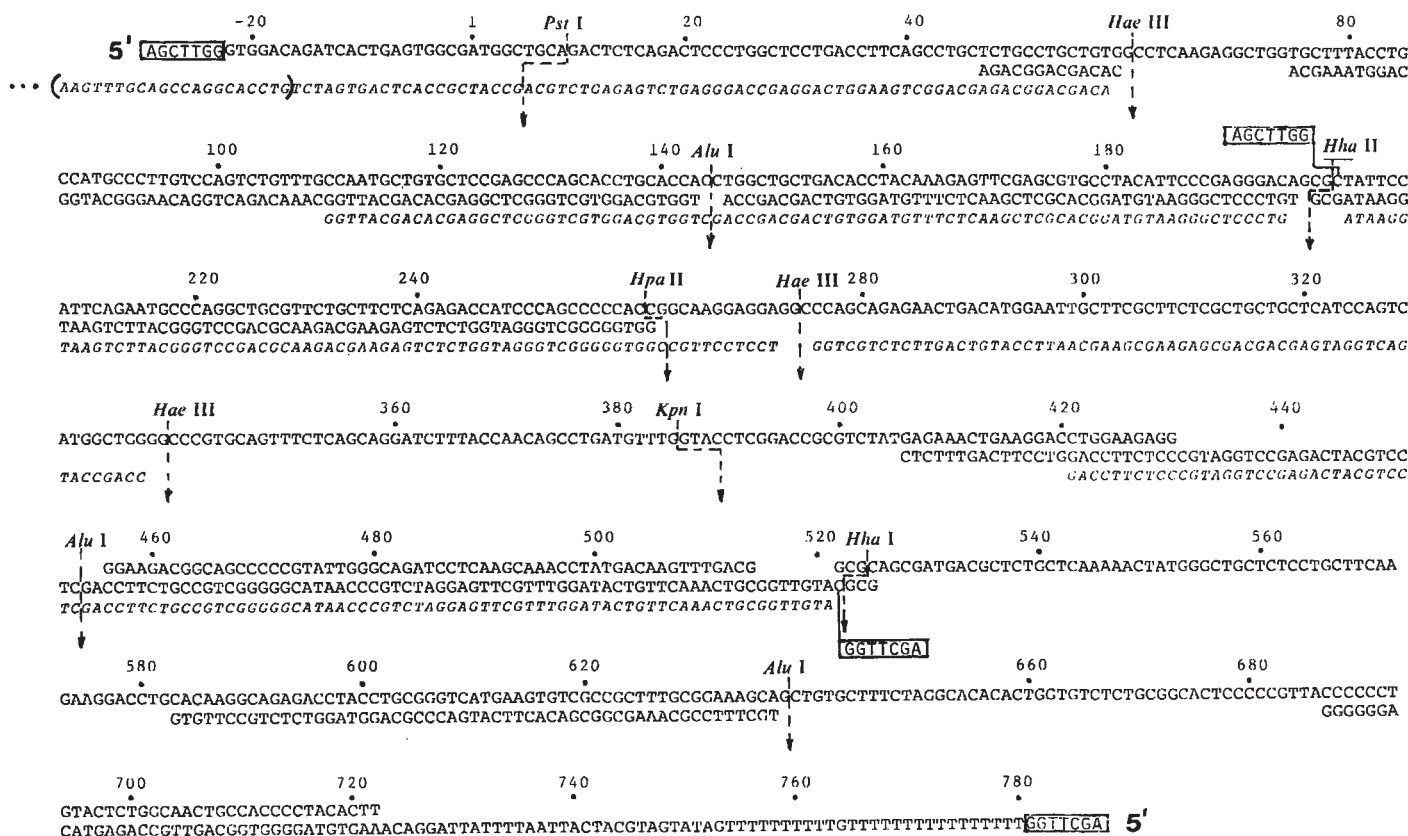


Fig. 7 Nucleotide sequence of the structural gene for rat growth hormone. The sequences shown were determined by the method of Maxam and Gilbert²⁵ from fragments of single-stranded cDNA to rat growth hormone mRNA and from the recombinant bacterial plasmids pRGH-1 and pRGH-2 (see Fig. 3). Also shown are the locations of restriction endonuclease sites used for cleaving cDNA and cloned DNAs. The sequences obtained from the single-stranded cDNA fragments, A₁, A₂, A₃, B₁, and B₂ are in italics and span residues 521–421, 272–202, 337–276, 196–110, and 58 to –37 respectively. (Position 1 is assigned to the A residue in the AUG initiator for the precursor protein.) The cDNA sequence in parentheses is not fully confirmed. Sequences obtained from cloned DNAs are shown in bold type. The second C residue in the sequence CC_TAGG appears as a gap in the sequencing gels due to methylation⁶³ in *E. coli* X1776. The presence of this nucleotide inferred from band spacings was confirmed from the sequence of the complementary strand or the cDNA. Nucleotides 523–525 were not directly sequenced but inferred from the existence of the *Hha*I site in this position. The boxed sequences represent the cohesive termini derived from the *Hind*III linker and mark the ends of pRGH-1 and pRGH-2.

The coding region contains 648 nucleotides which specify the 216 amino acids of the growth hormone precursor. The codon choices are listed in Table 1. It can be seen that codon utilisation is distinctly non-random, a phenomenon also noted in the coding sequences of other eukaryotic^{9,10,22,40} and prokaryotic^{47,48} mRNAs. As a consequence of such selective codon usage, growth hormone mRNA has a high G + C content (57.7%) which exceeds that of total rat DNA (43%)⁴⁹.

Amino acid sequence of rat pre-growth hormone

The established mRNA sequence permits the prediction of the complete amino acid sequence of the proposed physiological precursor form of rat growth hormone (pre-GH). The amino acid composition of this protein is given in Table 1.

The signal peptide of pre-GH (26 amino acids, MW 2,846) has a high content of hydrophobic amino acids (17 non-polar residues,

Table 1 Amino acid composition and codon choices of rat pre-growth hormone

Phe	UUU	6		Ser	UCU	0	(1)	Tyr	UAU	4		Cys	UGU	2	
	UUC	6	(1)		UCC	3			UAC	3			UGC	2	(1)
Leu	UUA	1			UCA	2		Ter	UAA	0		Ter	UGA	0	
	UUG	2			UCG	2			UAG	1		Trp	UGG	1	(2)
Leu	CUU	1		Pro	CCU	1	(1)	His	CAU	0		Arg	CGU	2	
	CUC	6	(2)		CCC	5	(1)		CAC	3			CGC	6	
	CUA	0			CCA	1		Gln	CAA	1	(1)		CGA	1	
	CUG	15	(4)		CCG	0			CAG	12	(1)		CGG	1	
Ile	AUU	3		Thr	ACU	1	(1)	Asn	AAU	2		Ser	AGU	1	
	AUC	5			ACC	7	(1)		AAC	3			AGC	6	(1)
	AUA	0			ACA	0		Lys	AAA	3		Arg	AGA	1	
Met	AUG	6	(1)		ACG	0			AAG	8			AGG	1	
Val	GUU	0		Ala	GCU	7	(3)	Asp	GAU	1		Gly	GGU	1	(1)
	GUC	2			GCC	8			GAC	9	(1)		GGC	3	
	GUA	0			GCA	1	(1)	Glu	GAA	4			GGA	1	
	GUG	2			GCG	2			GAG	10	(1)		GGG	3	

The amino acid sequence of rat pre-growth hormone has been solely predicted from the nucleotide sequence of rat growth hormone mRNA (see Fig. 8). Numbers next to codons indicate the number of amino acids using particular codons in the mature hormone, numbers in parentheses denote the extra amino acids of the pre-part. The N-terminal methionine is included. From a total of 216 codons used, 39.4% terminate in C, 34.3% in G, 18% in U, and 8.3% in A. The molecular weights of rat growth hormone and its precursor form as determined from the amino acid compositions are 2177.2 and 24622.6 respectively.

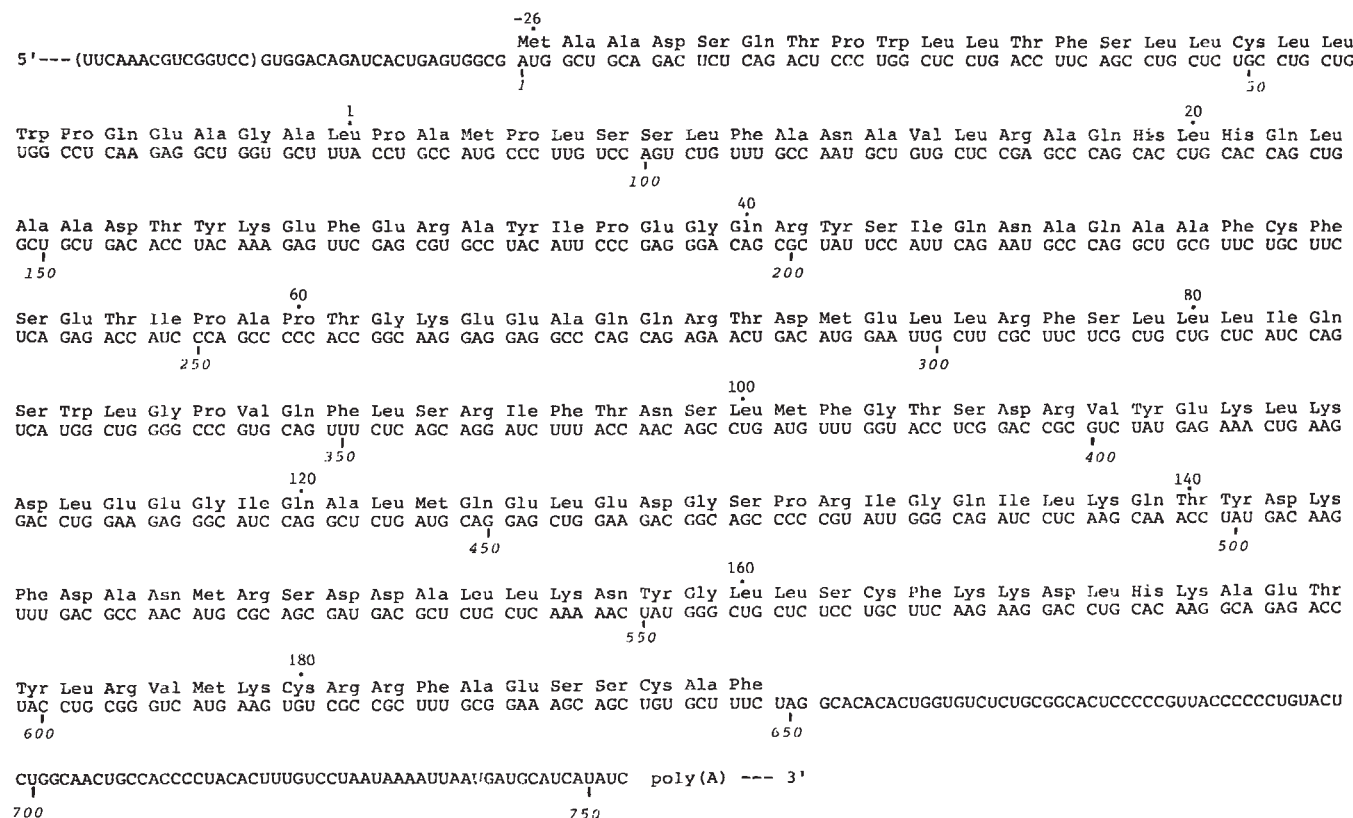


Fig. 8 Nucleotide sequence of rat growth hormone mRNA and amino acid sequence of rat pre-growth hormone. The nucleotide sequence of rat growth hormone mRNA has been deduced from the DNA sequences compiled in Fig. 7. The tentative 5' terminal sequence shown in parentheses is solely derived from cDNA fragment B₂ and does not allow the precise localisation of the 5' end of the mRNA. The RNA sequence permits prediction of the entire amino acid sequence for rat growth hormone (amino acids 1–190) and its pre-part (amino acids –26 to –1). The predicted sequence is in good agreement (except at positions 1 and 8) with the partial amino acid sequence of rat growth hormone as described by Wallis and Davies¹⁸ which comprises residues 1–43, 65–69, 108–113, 133–143, and 150–190.

six of which are Leu). A similar composition has been found in signal peptides of other proteins destined for secretion^{50,54}, and reflects their role in the attachment of ribosomes to the endoplasmic reticulum¹⁶. There is little apparent homology to other known signal peptides, except in the case of bovine growth hormone⁵⁴ where from the few established amino acid assignments at least residues –1, –9, –11, –14, –16, and –17 are in homologous positions in the rat and bovine precursors. The junction of the signal peptide with the mature rat hormone is provided by an Ala–Leu bond (see below) which is presumably cleaved by a membrane-bound peptidase to generate the N-terminus of growth hormone. Processing of secretory precursor peptides by cleavage after Ala residues has been observed in several other instances^{50,51,54}.

Rat growth hormone (RGH) itself comprises a peptide chain of 190 amino acids (MW 21,777) like the equine (EGH), bovine (BGH), and ovine (OGH) growth hormones. Compared with human growth hormone (HGH) and choriomatotomotropin (HCS) these four peptides contain two deletions (Phe and Ser at positions 44 and 108 in HGH) and one insertion (Phe at position 183)²⁶. The sequence of rat growth hormone (see Fig. 8) shows high but varying degrees of homology with the known sequences for HGH, BGH, OGH, and EGH²⁶. This finding reflects variable evolutionary rates of mammalian GHs, also noted by Wallis and Davies¹⁸. RGH differs from HGH by 34.4%, from BGH by 12.1%, and from EGH by 6.3%. In eight positions RGH uses amino acids not used in homologous positions in HGH, BGH, OGH, or EGH, although the observed changes are conservative in nature. The positions and amino acids are: 1, Leu for Phe; 70, Thr for Ser; 95, Ile for Val; 101, Met for Val; 124, Gln for Gly (HGH) or Arg (EGH, BGH, OGH); 133, Ile for Thr (HGH) or Ala (EGH, BGH, OGH); 146, Ala for Thr; 184, Ala for Val (HGH, EGH), or Gly (BGH, OGH).

The amino acid sequence of rat GH determined solely from DNA sequences is in good agreement with the partial protein sequence for this hormone obtained by Wallis and Davies¹⁸. The

only two differences are at positions 1 and 8 where our sequence reads Leu and Ser instead of Phe and Gly. Whereas a Ser at position 8 might have been expected because this amino acid is also used in EGH (which RGH is seen to follow more closely in sequence than BGH, OGH, or HGH), the presence of Leu in position 1 is unexpected since only Phe is used by any of the other known GHs. But, since allelic polymorphism has been observed in BGH⁵⁵, the Leu in position 1 may represent an allelic variant generated by a single base change (UUU → UUA). This variant could be expressed at a low level but may have been amplified in the cloning event. It is also possible that the Leu-containing peptide chain constitutes the only allele expressed in the cultured pituitary cells used as a source for GH mRNA. We are investigating this issue by determining the N-terminal residue of the peptide secreted by these cells.

This report demonstrates an analysis of the primary structure of a pituitary mRNA and the prediction of the amino acid sequence of the peptide product, pre-growth hormone. The techniques used (detection of low level nucleic acid sequences by enzymatic dissection of total cellular cDNAs⁹, rapid DNA sequencing²⁵, and molecular cloning^{64,65}) permit such an analysis to be expediently performed on a minimum amount of material, starting from a preparation that contains only a few per cent of the mRNA of interest.

The cloning of growth hormone DNA sequences should facilitate future work concerning the regulation of the growth hormone gene and will prove useful in developing systems for providing somatotropin, a hormone that is currently in short supply.

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Construction and analysis of recombinant DNA for human chorionic somatomammotropin

John Shine, Peter H. Seeburg, Joseph A. Martial, John D. Baxter & Howard M. Goodman

Department of Biochemistry and Biophysics, Metabolic Research Unit and Endocrine Research Division, School of Medicine, University of California, San Francisco, California 94143

DNA complementary to mRNA coding for the human polypeptide hormone, chorionic somatomammotropin, has been purified by specific restriction endonuclease digestion and religation before cloning into bacterial plasmids. The primary structure of a major portion of this mRNA species is deduced from the nucleotide sequence of the recombinant DNA.

THE polypeptide hormone human chorionic somatomammotropin (HCS, placental lactogen) is secreted by the placenta in increasing amounts during pregnancy, reaching levels of the order of 1 g per day in the last trimester¹. The physiological function of this hormone is still largely unknown, but it seems to act primarily on maternal metabolism, providing important nutrients of maternal origin necessary for foetal growth².

The primary structure of HCS is very similar to that of human growth hormone (HGH); both polypeptides contain 191 amino acids and are 85% homologous in amino acid sequence³. Such homology suggests that the genes for these two hormones, together with that coding for the related pituitary hormone prolactin, have evolved from a common ancestral gene^{3,4}. HCS, however, is secreted solely by the placenta, whereas growth hormone production is confined to the pituitary. This set of structurally related but differentially expressed genes thus forms an excellent system for investigating the structure, evolution and regulation of closely related eukaryotic genes.

In order to determine the degree of homology in the primary structure of these genes and to investigate the feasibility of

bacterial synthesis of human polypeptide hormones, we have constructed recombinant bacterial plasmids carrying DNA transcribed from HCS mRNA. We describe here the construction of these plasmids and the determination of the nucleotide sequence of the cloned DNA. Due to the stringent requirements of working with recombinant DNA from human sources⁵ we have developed a general approach for preparing homogeneous cDNA fragments transcribed from a complex mixture of mRNAs which should be applicable to many other mRNA species. A comparison of the primary structure determined for the coding region of HCS mRNA with that reported for rat growth hormone mRNA in the accompanying paper⁶ demonstrates the conservation of nucleotide sequences between these two mRNA species.

Purification of DNA complementary to HCS mRNA

Human placental RNA was isolated from a caesarian section placenta as previously described⁷ and the poly A-containing RNA separated by chromatography on oligo dT-cellulose⁸. The preparation was enriched for HCS mRNA by sucrose gradient sedimentation and pooling of the fractions (11–13S) which gave rise to a predominant 550 nucleotide cDNA *Hae*III fragment (see below). The major translation product of this RNA in a wheat germ cell free system is a 24,000 molecular weight (MW) polypeptide which accounts for approximately 50% of the total synthesis (results not shown). We have previously shown this polypeptide to be a precursor form of HCS by specific antibody precipitation⁷, suggesting that HCS mRNA constitutes about 50% of the total mRNA in this enriched preparation. The same polyadenylated RNA was used as a template for transcription into double-

stranded cDNA (see Fig. 1 legend). This cDNA was hetero-disperse as judged by polyacrylamide gel electrophoresis but gave rise to a predominant 550-base pair fragment when digested with the restriction endonuclease *Hae*III (Fig. 1, lane 1). We have previously demonstrated that this fragment is derived from DNA complementary to HCS mRNA by direct nucleotide sequencing of the corresponding single-stranded cDNA fragment⁷. As seen from Fig. 1, this fragment represents approximately 30% of the total cDNA synthesis, in agreement with the levels of HCS mRNA predicted from *in vitro* translation of the same RNA template.

Although unique cDNA fragments generated in this manner can be isolated from most of the other fragments by gel electrophoresis and characterised by direct sequence analysis, a significant percentage of the isolated material consists of contaminating sequences. In order to purify such cDNA fragments to near homogeneity we have developed a procedure involving specific cleavage and religation of the isolated DNA fragments and have used this approach to purify HCS cDNA as shown in Figs 1 and 2. A unique DNA fragment, generated as described above from a complex mixture of DNA fragments, is first treated with alkaline phosphatase (see below), isolated by gel electrophoresis (Fig. 1, lane 2) and then cleaved into two fragments with a second restriction endonuclease. The two major cleavage products are then re-isolated following separation by gel electrophoresis. During this step most of the contaminating sequences are removed as they will remain uncleaved or will be cleaved in a different relative position (Fig. 1, lane 3). The two isolated fragments are then religated to form the original fragment. Previous treatment of the original cDNA fragment with alkaline phosphatase ensures that these two fragments are capable of ligation to each other only in the correct orientation although self-ligation to form dimers also occurs (Fig. 1, lane 4). Isolation of the original fragment, reconstructed by ligation of its constituent digestion products, results in a DNA species which is greater than 99% homogeneous as judged by restriction endonuclease digestion and quantitation of the digestion products (Fig. 2). In order to isolate HCS cDNA for molecular cloning into bacterial plasmids, we followed the above approach using *Hha*I endonuclease to cleave the 550-base pair *Hae*III fragment as described in Figs 1 and 2.

Molecular cloning of HCS cDNA

The purified 550-base pair *Hae*III fragment was cloned into bacterial plasmids using chemically-synthesised restriction site 'linkers'¹¹⁻¹³. The self complementary decanucleotide (5)CCGAATTCGG(3') containing the *Eco*RI restriction endonuclease recognition and cleavage site was ligated to the HCS *Hae*III fragment (previously phosphorylated using ATP and T4 polynucleotide kinase to replace the 5' phosphates removed during purification) in a molar ratio of 50:1. Figure 3 (lane 2) shows that the *Eco*RI decamers ligated both to themselves and to the HCS cDNA. Digestion of these ligation products with *Eco*RI endonuclease results in cleavage at the *Eco*RI site of the decamers giving rise to HCS cDNA with *Eco*RI cohesive ends as well as cleaved decanucleotides (Fig. 3, lane 3). As the cleaved decamers also contain *Eco*RI termini and would compete with the cDNA for ligation to the similarly cleaved plasmid, the HCS cDNA was isolated by gel electrophoresis before ligation to the cloning vector.

The use of this decanucleotide for the cloning of the HCS cDNA fragment has the advantage that the original *Hae*III sites are recreated at each end of the DNA fragment, allowing its isolation from the plasmid in a form identical to that of the original fragment. This is particularly useful for further studies involving the use of the cloned HCS fragment as a primer for cDNA synthesis on placental mRNA or HnRNA.

For the cloning of the HCS cDNA we used the bacterial plasmid pMB9, a 3.5×10^6 MW molecule containing a single *Eco*RI site¹⁴. Infection of *E. coli* with pMB9 confers resistance to tetracycline. To select for recombinant plasmids we developed a method for ensuring that only plasmids containing an inserted

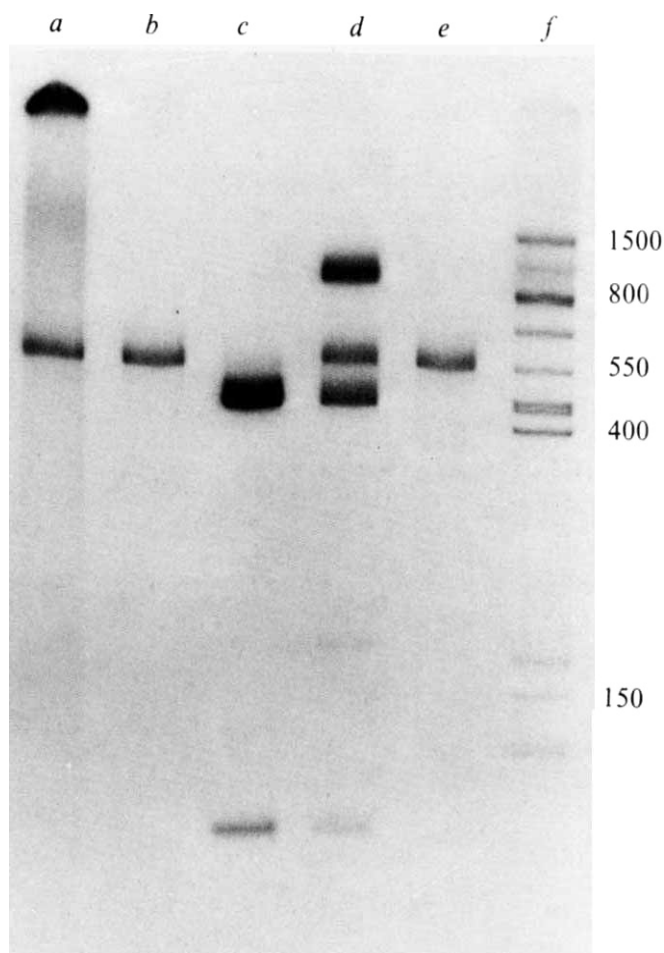


Fig. 1 Purification of HCS cDNA. Polyadenylated placental RNA isolated as previously described⁷ was enriched for HCS mRNA by sedimentation in a 5–20% sucrose gradient at 4 °C in the SW 27 rotor at 25,000 r.p.m. for 16 h. The 11–13S region of the gradient was pooled and 100 µg of this RNA used for the synthesis of double-stranded cDNA as described elsewhere⁶. DNA synthesis was stopped by extraction with phenol-chloroform and the DNA precipitated with ethanol. Digestion of the cDNA with *Hae*III endonuclease (a gift from W. Brown) was carried out in 50 µl 6 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 6 mM β-mercaptoethanol with 2 units of *Hae*III at 37 °C for 2 h, after which 0.1 units of bacterial alkaline phosphatase (Worthington, BAPF) were added and digestion continued at 60 °C for 10 min. After phenol-chloroform extraction, the DNA was precipitated with ethanol, dissolved in 20 µl 10 mM Tris-HCl, pH 8, 1 mM EDTA and electrophoresed in a 6% polyacrylamide gel. The 550-base pair fragment was excised from the gel, eluted electrophoretically and digested with 4 units of *Hha*I endonuclease (New England Biolabs) at 37 °C for 2 h in 50 µl of the same buffer used for digestion with *Hae*III endonuclease. The digestion products were then separated by electrophoresis in a 6% polyacrylamide gel and the two fragments eluted electrophoretically. The eluted fragments were combined and re-ligated in a 20 µl 66 mM Tris-HCl, pH 7.6, 9 mM MgCl₂, 15 mM dithiothreitol, 1 mM ATP containing 20 µg ml⁻¹ of T₄ DNA ligase⁹ at 15 °C for 2 h, diluted to 200 µl with 0.1 M NaCl, extracted with phenol-chloroform and the DNA precipitated with ethanol. The ligation products were dissolved in 20 µl 10 mM Tris-HCl, pH 8, 1 mM EDTA, separated by electrophoresis in a 6% polyacrylamide gel and the 550-base pair fragment excised and eluted electrophoretically. This fragment was used in the cloning experiments. Samples from each step in the purification procedure were analysed by electrophoresis in a 6% polyacrylamide gel in 50 mM Tris-borate, pH 8, 1 mM EDTA at 100 V for 2 h. After electrophoresis the gel was dried and exposed to X-ray film (Kodak NS2T) to visualise the labelled fragments. *a*, *Hae*III digest of total placental cDNA; *b*, the 550-base pair HCS *Hae*III fragment isolated from (*a*); *c*, *Hha*I digest of the isolated *Hae*III fragment; *d*, re-ligation of the separately isolated *Hha*I digestion products; *e*, the 550-base pair fragment regenerated by ligation of its constituent *Hha*I fragments after isolation from a 6% polyacrylamide gel; *f*, ³²P-labelled *Hpa*II digest of ds M13 DNA used as size markers.

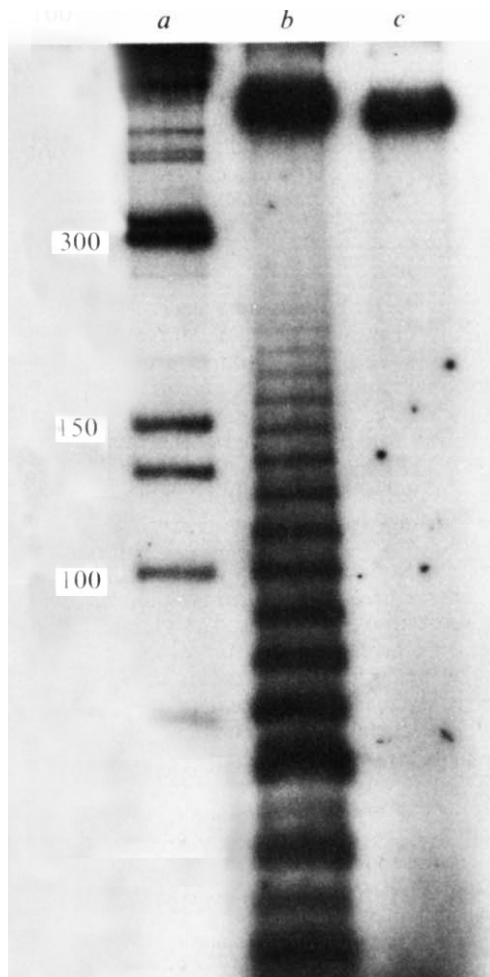
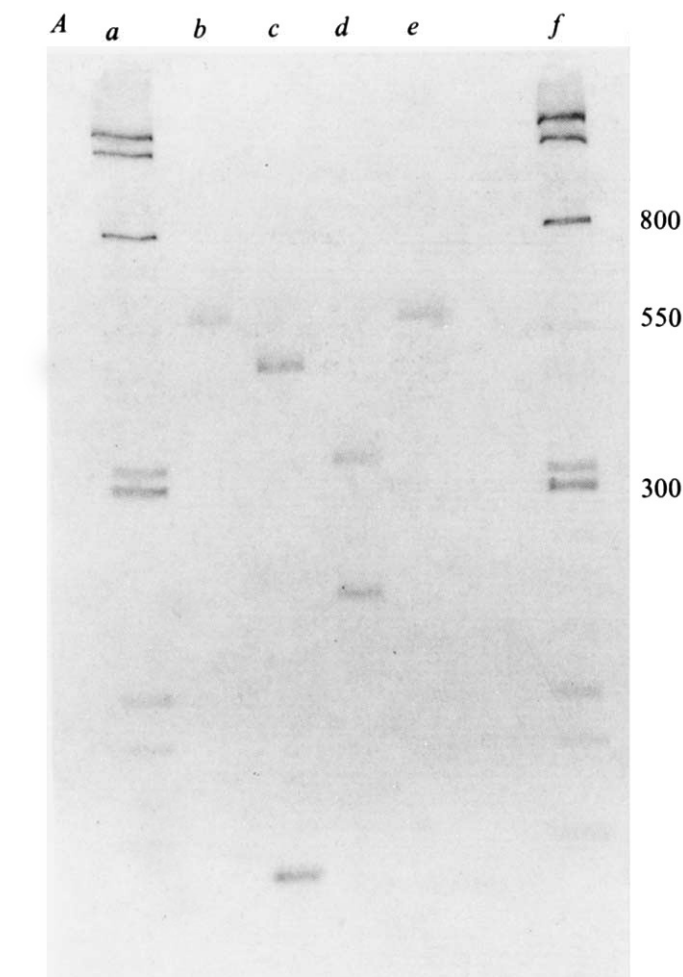


Fig. 3 Ligation of purified HCS cDNA to *Eco*RI decamers. Purified HCS cDNA *Hae*III fragment was phosphorylated at its 5' termini (see text) using T_4 polynucleotide kinase and ATP¹⁰. *Eco*RI decamers (5'-CCGAATTCGG-3'; a gift from R. H. Scheller) were then ligated to the fragment in a molar ratio of approximately 50:1 in 50 μ l of 66 mM Tris-HCl, pH 7.6, 9 mM MgCl₂, 15 mM dithiothreitol, 1 mM ATP and 20 μ g ml⁻¹ T_4 DNA ligase⁹. After ligation at 4 °C for 18 h, the reaction was stopped by extraction with phenol-chloroform. The ligation products were precipitated with ethanol, redissolved in 50 μ l 100 mM NaCl, 50 mM Tris-HCl, pH 7.6, 7 mM MgCl₂, and digested with 50 units *Eco*RI endonuclease at 37 °C for 2 h. Samples were analysed on an 8% polyacrylamide gel as described in the legend to Fig. 1. *a*, ³²P-labelled *Hae*III digest of ds M13 DNA; *b*, ligation of *Eco*RI decamers to the purified HCS *Hae*III fragment; *c*, *Eco*RI endonuclease digest of the ligation products shown in *b*.

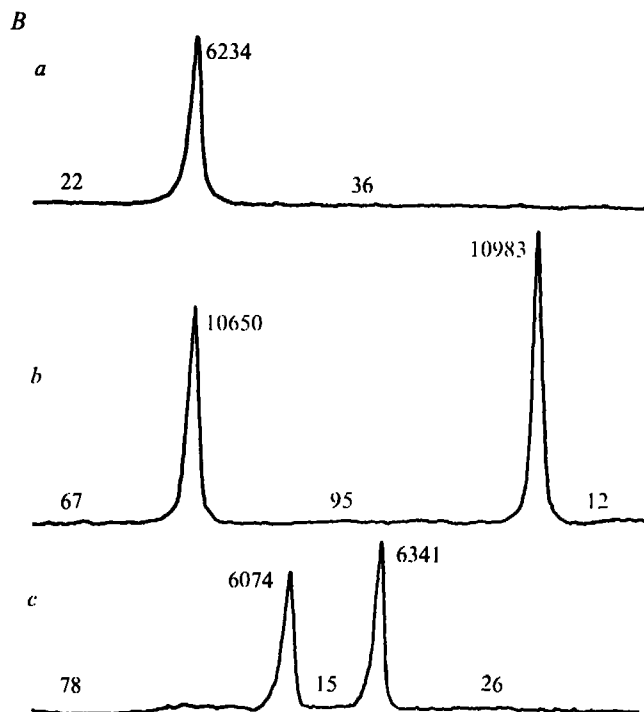


Fig. 2 Purity of HCS cDNA used for molecular cloning. The isolated HCS cDNA *Hae*III fragment, purified as described in Fig. 1, was digested with either *Hha*I or *Hpa*II (New England Biolabs) in 50 μ l 6 mM Tris-HCl, pH 7.6, 6 mM MgCl₂, 6 mM β -mercaptoethanol at 37 °C for 2 h and terminally labelled using [γ -³²P]ATP and T_4 polynucleotide kinase¹⁰. The labelled digestion mix was extracted with phenol-chloroform and the DNA separated from unincorporated [γ -³²P]ATP by chromatography on Sephadex G-50. The labelled fragments were precipitated with ethanol, resuspended in 20 μ l 10 mM Tris-HCl, pH 8, 1 mM EDTA and subjected to electrophoresis in a 6% polyacrylamide gel. Following electrophoresis the gel was exposed to X-ray film to visualise the labelled fragments. *A*: *a* and *f*, ³²P-labelled *Hae*III digest of ds M13 DNA; *b* and *e*, purified HCS cDNA *Hae*III fragment; *c*, *Hha*I digest of the HCS cDNA *Hae*III fragment; *d*, *Hpa*II digest of the HCS cDNA *Hae*III fragment. *B*: Scan of the autoradiogram shown in (*A*). *a*, Purified HCS cDNA *Hae*III fragment; *b*, *Hha*I digest of the HCS cDNA *Hae*III fragment; *c*, *Hpa*II digest of the HCS cDNA *Hae*III fragment. The purified HCS cDNA *Hae*III fragment was judged to be greater than 99% homogeneous by quantitation of the distribution of radioactivity in each of the two restriction endonuclease digests. Similar results were obtained by analyses of an *Xba*I digest of the purified cDNA fragment. Numbers represent c.p.m. in each fragment and in the intervening regions of the gel.

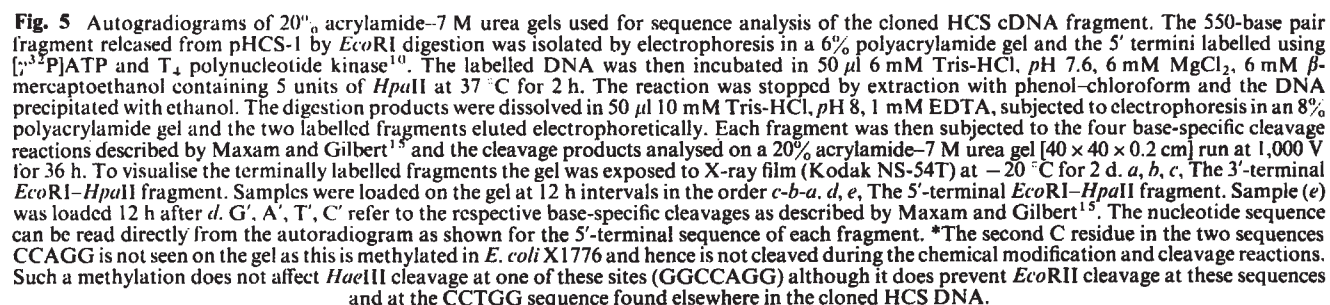
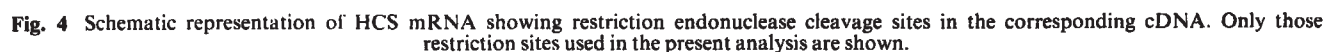


Fig. 9 Conservation of nucleotide sequences in the coding region of HCS and rat growth hormone mRNAs. Two portions of the mRNA coding region of HCS (amino acids 77-96 and 128-147) and rat growth hormone (RGH) (76-95 and 126-145) are shown where the homology in amino acid sequence is high. Silent nucleotide changes (which do not result in an amino acid change) are boxed.

HCS----	Arg	Ile	Ser	Leu	Leu	Leu	Ile	Glu	Ser	Trp	Leu	Glu	Pro	Val	Arg	Phe	Leu	Arg	Ser	Met
	CGC	AUC	UCG	CUG	CUG	CUC	AUC	GAG	UCG	UGG	CUG	GAG	CCC	GUG	CGG	UUC	CUC	AGG	AGU	AUG
RGH----	CGC	UUC	UCG	CUG	CUG	CUC	AUC	CAG	UCG	UGG	CUG	GGG	CCC	GUG	CAG	UUU	CUC	AGC	AGG	AUC
	Arg	Phe	Ser	Leu	Leu	Leu	Ile	Gln	Ser	Trp	Leu	Gly	Pro	Val	Gln	Phe	Leu	Ser	Arg	Ile
HCS----	Leu	Glu	Asp	Gly	Ser	Arg	Arg	Thr	Gly	Gln	Ile	Leu	Lys	Gln	Thr	Tyr	Ser	Lys	Phe	Asp
	CUG	GAA	GAC	GGC	AGC	CGC	CGG	ACU	GGG	CAG	AUC	CUC	AAG	CAG	ACC	UAC	AGC	AAG	UUU	GAC
RGH----	CUG	GAA	GAC	GGC	AGC	CCC	CCU	AUU	GGG	CAG	AUC	CUC	AAG	CAG	ACC	UAC	GAC	AAG	UUU	GAC
	Leu	Glu	Asp	Gly	Ser	Pro	Arg	Ile	Gly	Gln	Ile	Leu	Lys	Gln	Thr	Tyr	Asp	Lys	Phe	Asp

produced by *AluI*, *HhaI* or *HpaII* endonuclease cleavage of the DNA (Fig. 6). By comparison with the known amino acid sequence of HCS, the 557 nucleotide sequence shown in Fig. 7 represents that portion of the coding region of HCS mRNA from amino acids 24-191, plus 50 nucleotides of the 3'-untranslated region. The amino acid sequence predicted from the nucleotide sequence is identical with that previously published³ confirming the validity of amino acid sequence determination by nucleotide sequence analysis.

The fidelity of cDNA cloning in bacterial plasmids is seen from the presence of identical sequences in the cloned DNA and those previously determined directly by cDNA sequencing⁷. This is also shown in the accompanying paper on rat growth hormone mRNA⁶ and demonstrates the validity of using recombinant DNA for structural studies of eukaryotic genes and will allow examination of the fidelity of expression of these genes in bacteria.

Primary structure of HCS mRNA

An analysis of the coding region of HCS mRNA indicates that codon utilisation is non-random with certain strongly preferred codon choices for some of the amino acids (Fig. 8). A very similar pattern of codon utilisation is seen in rat growth hormone mRNA⁶ which is reflected in the conservation of sequences found between these two mRNA species (see below). Preferential codon utilisation, which is clearly different from that seen in bacteriophage and SV40 mRNAs¹⁶⁻¹⁸, has also been previously observed

Phe	UUU	2	Ser	UCU	2	Tyr	UAU	4	Cys	UGU	1
	UUC	8		UCC	5		UAC	4		UGC	3
Leu	UUA	-		UCA	1	Term.	UAA	-	Term.	UGA	-
	UUG	-		UCG	4		UAG	1	Trp	UGG	1
Leu	CUU	-	Pro	CCU	-	His	CAU	2	Arg	CGU	-
	CUC	7		CCC	2		CAC	2		CGC	4
	CUA	3		CCA	1	Gln	CAA	2		CGA	-
	CUG	11		CCG	1		CAG	7		CGG	2
Ile	AUU	2	Thr	ACU	1	Asn	AAU	1	Ser	AGU	1
	AUC	5		ACC	5		AAC	6		AGC	4
	AUA	-		ACA	3	Lys	AAA	1	Arg	AGA	-
Met	AUG	5		ACG	2		AAG	8		AGG	3
Val	GUU	-	Ala	GCU	-	Asp	GAU	1	Gly	GGU	-
	GUC	1		GCC	2		GAC	13		GGC	4
	GUA	-		GCA	1	Glu	GAA	5		GGA	-
	GUG	4		GCG	-		GAG	8		GGG	3

Fig. 8 Codon utilisation in HCS mRNA. Term, termination signal.

in rabbit β -globin mRNA¹⁹ and rat insulin mRNA¹³. The use of certain codons (for example, CUC and CUG for Leu, GAC for Asp, AAG for Lys) seems to be favoured in all eukaryotic cellular mRNAs examined to date, while others are apparently strongly selected against (for example, UUA for Leu, AUA for Ile, GUA for Val). Such codon selection reflects the apparent preference for C or G in the third position of eukaryotic codons. In the coding region of HCS mRNA, 75 codons (46%) terminate in C, 53 (33%) in G (excluding AUG and UGG, which only have one codon), but only 17 (10%) end in U and 17 in A (10%) (Fig. 8).

Comparison of the coding regions of HCS and rat growth hormone mRNA demonstrates the conservation of nucleotide sequences between these two mRNA species with very few 'silent'

codon changes (Fig. 9), supporting the suggestion that the structural genes for chorionic somatomammotropin and growth hormone have evolved by duplication of a common ancestral gene. Such sequence conservation between related mRNAs from diverse organisms is likely to result from several factors, including selection of a favoured mRNA structure and the availability of specific isoaccepting tRNAs.

The 3'-untranslated region of HCS mRNA is characterised by a large palindromic sequence (5'-GUGACCCCUCCCC-AGUG-3') previously seen from direct sequence analysis of cDNA fragments⁷. In contrast to the coding region, most of the 3'-untranslated region of rat growth hormone mRNA shows very little homology with the region determined in HCS mRNA although a somewhat similar palindromic-type sequence containing oligo C tracts (5'-UCCCCCGUUACCCCCCU-3') is found in a corresponding position in rat growth hormone mRNA⁶. Other palindromic sequences are also present in a similar position in the 3'-untranslated region of human α -globin mRNA (5'-UCCUCCCCUCCU-3')²⁰ and human β -globin mRNA (5'-GGGGGAUUAUAUGAAGGG-3')²¹. The function of such symmetrical sequences is not known, but they may represent recognition sites for specific protein-nucleic acid interactions involved in the regulation of transcription, translation or processing of the mRNA.

Amplification in bacterial plasmids of HCS gene sequences provides a probe to study regulation of this gene (and the closely related growth hormone gene) in normal and pathological states. The fidelity shown in the replication of these sequences in bacteria will also allow investigation of the expression of eukaryotic structural gene sequences when linked to bacterial promoters in a manner defined at the molecular level.

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letters to nature

Black holes in closed universes

BLACK holes are now the subject matter of at least half the papers in general relativity. These papers rest on a foundation of sand, for black holes have been successfully defined only in asymptotically flat spacetimes, and no one really believes that the Universe is asymptotically flat. I shall provide a firm basis for black hole physics in the present paper; I shall extend the concept of 'black hole' to arbitrary stably causal spacetimes by essentially defining a black hole to be that object which contains all the 'small' trapped surfaces. As for most astrophysical applications black hole surfaces are located approximately by the outermost trapped surface boundary, this new definition allows results which depend only on the local behaviour of black holes in asymptotically flat spacetimes to be extended (approximately) to closed universes. Results which depend on the global behaviour of black holes cannot in general be extended to closed universes. For example, it is shown that the black hole area theorem—the statement that black holes never decrease their cross-sectional area—cannot be extended to closed universes. The new concept of black hole yields a purely geometrical definition of time direction in closed universes. My notation will be that of ref. 1.

Definition: a black hole is the closure of the topologically smallest future set I^+ such that (a) I^+ contains all non-cosmological trapped surfaces; (b) the boundary of I^+ is generated by null geodesic segments which are boundary generators of TIPs.

(In the topological sense, a set A_1 is the smallest member of $\{A_i\}$ if $A_1 \subseteq A_i$ for all i .) A black hole as defined above is a 4-dimensional object. A 3-dimensional black hole—a concept useful for discussing black hole collisions—can be obtained by intersecting the black hole defined above with a partial Cauchy surface. This is analogous to the procedure used in asymptotically flat spacetimes to obtain a 3-dimensional black hole (ref. 1, p 315).

The distinction between cosmological and non-cosmological trapped surfaces can be clarified by comparing the spherical trapped surfaces in Schwarzschild spacetime with the spherical trapped surfaces appearing in the contracting phase of a closed Friedmann universe². In both cases the boundary of the trapped surface region T is a marginally trapped surface ∂T with $1\chi_{ab}g^{ab} = 0$ and $2\chi_{ab}g^{ab} < 0$. (That is, one family of null geodesics orthogonal to ∂T has zero divergence at ∂T , while the other family has negative divergence at ∂T . $1\chi_{ab}$ is a null second fundamental form of ∂T .) In the Schwarzschild case the family with $1\chi_{ab}g^{ab} < 0$ points toward the trapped surfaces defining ∂T , while in the Friedmann case the $1\chi_{ab}g^{ab} < 0$ family points away from the trapped surfaces. I propose to make this distinction general.

Definition: for any stably casual spacetime (M, g) , let ∂T be a marginally trapped surface such that ∂T is part of the boundary of a continuous set T of acausally related trapped surfaces, and $1\chi_{ab}g^{ab} < 0$, $2\chi_{ab}g^{ab} = 0$. If the family of null geodesics defined by the form $1\chi_{ab}$ point in the direction of the trapped surfaces T , then any element of T together with any trapped surface constructed by continuously deforming an element of T so that each element of the deformation is a trapped surface and is acausal with respect to all elements of T , will be called a non-cosmological trapped surface. Trapped surfaces in (M, g) which cannot be generated by the above procedure will be considered cosmological.

Recall (ref. 1, p 315) that a black hole is defined in an asymptotically flat spacetime (M, g) to be set $M - J^-(I^+) \equiv B$. It is known that this set contains all trapped surfaces (ref. 1,

p 311), and that its boundary is generated by the boundary generators of TIPs. It can be shown that if B arises to the future of a Cauchy surface and approaches a Kerr–Newman black hole limit, then the new definition is equivalent to the old. The details of this result will be published elsewhere.

John Wheeler is the most ardent proponent of the one-cycle closed universe³. I shall make his conception of cosmology precise by defining a Wheeler Universe to be a globally hyperbolic spacetime which is closed in space (the Cauchy surfaces are compact), and closed in time (all inextendible null geodesics are past and future incomplete and $d(M, M)$ is finite). It is generally believed⁴ that in the physically realistic case, an incomplete causal geodesic terminates in a singularity which destroys everything which enters it. This type of singularity I have termed⁵ a 'strong curvature singularity'—more precisely, a point p on the b -boundary is said to be such a singularity if for every causal geodesic $\lambda(t)$ which intersects p , all linearly independent vorticity-free Jacobi fields along $\lambda(t)$ which are normal to the tangent vector of $\lambda(t)$ define a volume (or area) element which vanishes as λ approaches p .

Thus it is expected that a physically realistic closed universe would be a Wheeler universe which begins and ends in strong curvature singularities. I will now show that black holes in physically realistic closed universes can violate the black hole area theorem.

Theorem: let (M, g) be a Wheeler universe for which all points on the b -boundary are strong curvature singularities. Let B be a black hole such that the null geodesic generators of ∂B all have a past endpoint at the same point $p \in M$. If the null convergence condition holds, then B does not satisfy the black hole area theorem.

Proof: there exists a Cauchy surface S_1 with $p \ll S_1$ and $J^+(p) \cap S_1$ compact. Since all generators of ∂B intersect p , and since ∂B and $J^+(p)$ are both closed and without boundary, we must have $\partial B = J^+(p)$. Hence $\partial B \cap S_1$ is compact. The divergence θ of the null geodesic generators of ∂B varies continuously on $\partial B \cap S_1$. Furthermore, since ∂B is achronal, θ can have the value $-\infty$ only at the point p on any generator γ . Now θ satisfies the same equation as the expansion of the area defined by the vorticity-free linearly independent Jacobi fields along γ . Hence θ approaches $-\infty$ as the future limit of the affine parameter is approached, since γ terminates in a strong curvature singularity and the area defined by linearly independent Jacobi fields vanishes if and only if θ approaches $-\infty$ (ref. 1, p 100). Since the null convergence condition holds, once θ becomes negative, it remains negative for the remainder of γ 's future history. Since (M, g) is globally hyperbolic, $M = S \times R^1$; we can foliate M with Cauchy surfaces $S(\tau)$, and we can choose $S_1 = S(\tau_1)$. By an argument similar to that of proposition 7.24 of ref. 6, the point on ∂B at which θ of a geodesic generator first becomes negative varies continuously with the generator γ . Let t_0 be the affine parameter distance to the future of $\partial B \cap S_1$ along a generator $\gamma(t)$ at which θ first becomes negative, with $t_0 = 0$ if θ is negative at $\partial B \cap S_1$. t_0 is thus a continuous function on the compact set $\partial B \cap S_1$, and so it achieves its upper bound. Hence there is a Cauchy surface $S(\tau_2)$ to the future of $S(\tau_1)$ for which θ is negative along every null geodesic generator of ∂B at every point in $\partial B \cap J^+(S(\tau_2))$. Thus the cross-sectional area of ∂B decreases, and this contradicts the black hole area theorem, which says the area never decreases.

A spherically symmetric black hole which forms by gravitational collapse in a closed Friedmann universe would satisfy the conditions of the above theorem if the matter tensor were chosen

so that the global hyperbolicity requirement and the b-boundary condition were obeyed^{7,8}.

Many authors (for example, ref. 9) have discussed the connection between Time's Arrow and black holes. The general consensus seems to be that one obtains a preferred direction of time only in those universes for which the initial conditions allow more black holes than white holes. If this connection between entropy increase and black holes is correct, then it is possible to use the new concept of black hole to define the direction of time geometrically—no reference to the behaviour of matter is made.

Definition: let (M, g) be a stably causal spacetime. The future time direction of (M, g) will be the direction of time for which the volume (in the metric g) of the region generated by all non-cosmological trapped surfaces is greatest.

This definition assumes, of course, that the volume occupied by the black holes is finite at least in one time direction. This is clearly true in Wheeler universes which begin and end in strong curvature singularities, for in this case the total volume of spacetime is finite. The relationship between the above geometrical time direction and the directions of time as defined by other, non-gravitational, physical processes will be discussed elsewhere.

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FRANK J. TIPLER

Department of Mathematics,
University of California at Berkeley,
Berkeley, California 94720

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Detection of H₂O emission from galaxy NGC253

The 6₁₆–5₂₃ transition of water vapour at 22.235 GHz was observed for the first time in an external galaxy by Churchwell *et al.*¹ in M33 with the 100-m Effelsberg radio telescope in late 1976, after some previous unsuccessful searches^{2,3}. We report here the second detection of H₂O emission from an external galaxy, NGC253, a large edge-on spiral galaxy situated at 3.4 Mpc (ref. 4), about five times farther away than M33. Previous observations of other molecules pointed NGC253 as a good candidate for H₂O emission: OH was detected by Weliachew⁵ and confirmed by other workers^{6,7}; CO was detected by Rickard *et al.*⁸ and by Solomon and Zafra⁹, and H₂CO was detected by Gardner and Whiteoak¹⁰. The time schedule of the 13.7-m Itapetinga radio telescope during 1977 May permitted a very long integration to be performed on this object, which is observable more than seven hours a day at elevation angles greater than 30°. The observations were made with a double sideband balanced mixer receiver of about 1,000 K system temperature and a 46 channel, 100 kHz resolution filter bank. Beam switching at a frequency of about 100 Hz was used, the main beam and then the 9' reference beam being pointed at the nucleus of NGC253 for alternate intervals of 1 min. The sign of the recorded signal was changed every minute by the data acquisition computer, so that possible zero-level offsets of the channels were eliminated. One-hour integrations were made with the filter bank centred alternatively

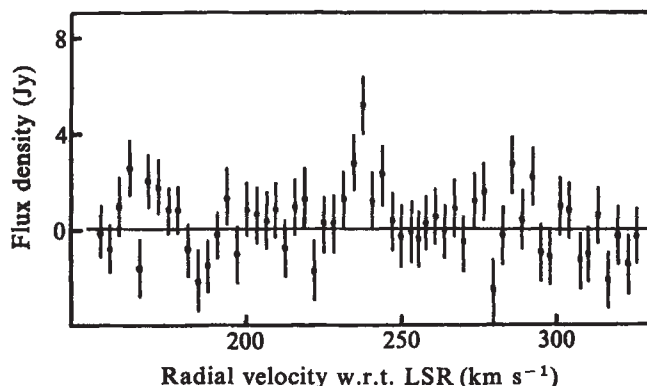


Fig. 1 The H₂O (6₁₆–5₂₃) spectrum of NGC253. The error bars are equal to twice the standard error of the mean obtained for each channel. The continuum flux of the nucleus of NGC253 has already been removed from the spectrum obtained from observations.

at three different frequencies in order to obtain greater velocity coverage, until a total on-source integration time of 21 h was completed at each frequency band. The data analysis program corrected the antenna temperatures for atmospheric attenuation as a function of zenith angle $\exp(-\tau \sec z)$ and then investigated the distribution of the results of the one-hour observations for each channel, giving the standard error of the mean and rejecting data more than 3σ different from average values; the channels were subsequently averaged two by two, simulating a 200 kHz resolution system.

The H₂O spectrum of NGC253 is presented in Fig. 1, with error bars representing twice the standard error of the mean. An emission feature of 5.2 ± 1.2 Jy appears at $v = 233$ km s⁻¹, this is possibly associated with the same emitting region which produces the 1667 MHz OH emission observed by Gardner and Whiteoak at 239 km s⁻¹ (246 km s⁻¹ heliocentric) as such small velocity differences between OH and H₂O peaks are common in the sources of our galaxy, while the total velocity range due to rotation in NGC253 is larger than 200 km s⁻¹. We have no precise information on the position of the H₂O source since the 4' beam is an important fraction of the 23' × 5' galaxy. The intrinsic power of the H₂O emission at 233 km s⁻¹ is about three times greater than that of W49, the strongest H₂O source of our galaxy, during its maxima. The H₂O emission is, however, not so anomalous as the OH emission, as Gardner and Whiteoak estimated for the OH emission at 239 km s⁻¹ an output power about two orders of magnitude greater than that of the most powerful Class I OH sources.

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JACQUES R. D. LÉPINE
PAULO MARQUES DOS SANTOS

CRAAM/ON/CNPq—Conselho Nacional de
Desenvolvimento Científico e Tecnológico,
Rua Ceará no. 290, 01243—São Paulo, SP, Brazil

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Detection of intergalactic gas in distant, rich clusters

WE have detected a reduction in intensity or 'cooling' of the cosmic microwave background as this radiation passes through several rich clusters of galaxies. These observations imply the presence of substantial quantities of hot intergalactic gas in the clusters observed. The 'cooling' effect, due to inverse Compton scattering of the microwave photons by hot intergalactic gas in the clusters, was predicted earlier by Sunyaev and Zel'dovich¹. The fractional change in the intensity of the microwave background is given by

$$\frac{\Delta I}{I} = \frac{x e^x}{e^x - 1} \left[\frac{x}{\tanh(x/2)} - 4 \right] \int_0^\tau \frac{k T_e}{m_e c^2} d\tau \quad (1)$$

where x is related to the observing frequency by $x = h\nu/kT_r$, with T_r the temperature of the microwave background, taken to be 2.7 K; τ is the total optical depth for Thomson scattering through a cluster; and m_e and T_e are the electron mass and electron temperature of the hot gas. Gull and Northover², observing at $\lambda = 3$ cm, have recently reported evidence for this effect in several clusters. We have tried to confirm their work at somewhat higher sensitivity, and at a shorter wavelength. A shorter wavelength was chosen to lessen interference and confusion by radio sources in or near the clusters observed. Our results on eight clusters which are Uhuru X-ray sources will be reported elsewhere³. In general, we were able to set 2σ limits of $\lesssim 4 \times 10^{-4}$ K on the reduction in temperature of the cosmic background; in some cases these limits lie below the results reported by Gull and Northover. We do see statistically significant evidence for the 'cooling' effect in three clusters, however—all of which are in Abell⁴ richness class 4. In the case of one of the three, Abell 2218, our results confirm the measurement of $\Delta T = -1.94 \pm 0.54$ mK by Gull and Northover². Here we call attention to the apparent strong correlation between the richness class and the detectability of the inverse

aperture and beam efficiency³, using a figure of 40%. We note that each of these values of ΔT is larger in magnitude than any ΔT measured for nearby, but less rich, clusters such as Coma or Abell 576.

Four corrections to the tabulated results are necessary before they can be used to draw inferences about the amount and distribution of ionised matter in these clusters. First, the observed ΔT values are expressed in antenna temperature, and must be converted to differences in thermodynamic temperature for comparison with the 2.7 K background. The correction factor is 1.027. This reduction in the sensitivity of the results is more than off-set by an increase in the magnitude of the function of x in equation (1). With these two corrections applied, we find

$$\int_0^\tau \frac{k T_e}{m_e c^2} d\tau = -0.15 \Delta T$$

Next, the antenna pattern must be convolved with the surface brightness distribution of the source. The latter, of course, is model dependent. If we adopt, for instance, the adiabatic model of Sarazin and Bahcall⁵, with $T_e = 10^8$ K, $H_0 = 55$ km s⁻¹ per Mpc, and a cluster core radius of 0.25 Mpc, we find the values of the average proton number density given in Table 1. Fourth and finally, a correction should be made for the finite separation of our beams (9.0') compared to the cluster radii. For the Sarazin and Bahcall models, however, this correction can be neglected for these distant clusters.

The large size of the microwave effect and the inferred values of n_0 suggest that these clusters should be luminous X-ray sources¹¹. That they are not included in the 4U catalogue is no doubt due to their distance: all are Abell distance class 6. Our results do suggest, however, that these rich clusters—despite their distance—should be readily detected by the next generation of X-ray satellites, and that they may prove to be among the most luminous X-ray clusters.

GEORGE LAKE

Physics Department,
Princeton University,
Princeton, New Jersey

R. B. PARTRIDGE

Haverford College,
Haverford, Pennsylvania

Received 4 August; accepted 19 October 1977.

Table 1 Richness class 4 clusters observed. The measured changes in temperature, with their associated standard deviations of the mean, are given. The approximate values of the proton density in the clusters are derived under the assumptions listed in the text.

Abell cluster	α_{1950}	δ_{1950}	(ΔT mK)	Inferred n_0 (10^{-3} cm ⁻³)
1689	13h08m58s	-01° 06.5'	-1.06 ± 0.46	5 ± 2
2125	15h40m26s	+66° 28.8'	-3.10 ± 0.34	14 ± 1.5
2218	16h35m43s	+66° 19.5'	-2.65 ± 0.23	12 ± 1

Compton 'cooling'. These results may be of interest to those constructing models for the intergalactic plasma in clusters⁵⁻¹¹, especially when the results are combined with the more sensitive X-ray observations we can expect if the new HEAO satellites are successful.

Our measurements were made at $\lambda = 9$ mm at the Cassegrain focus of the 11-m NRAO* telescope in Tucson, Arizona, during excellent weather in April 1977. The National Radio Astronomy Observatory is operated by Associated Universities, Inc., under contract with the NSF.) At the time we used them, the dual receivers had system temperatures of ~ 560 K (double sideband), with $\Delta\nu = 10^9$ Hz. The telescope beam was 3.6' full width at half-power; we employed beam switching in azimuth with a beam throw of 9.0'.

Three richness class 4 clusters were observed for periods of 5–9 h; 'cooling' of the microwave background was detected in all of them. The observed changes in temperature and the associated standard deviations of the means are shown in Table 1. All data were weighted equally in forming these means. The values shown in Table 1 have been corrected for atmospheric extinction, and for

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Radio polarisation of quasars with optical absorption spectra

CONWAY and Gilbert¹ have noted that the mean linear polarisation at wavelength λ 49 cm of highly redshifted quasars is significantly less for those with optical absorption lines compared with those with none. They suggested that the cause was Faraday depolarisation in the same regions responsible for the absorption lines. Gardner and Whiteoak² pointed out similar differences between the two quasar types at λ 11 cm and 6 cm. They regarded lower intrinsic polarisation of absorption-line quasars as a more likely explanation. Both findings were based on extremely small statistical samples. Subsequently much more data have become available at both optical and radio wavelengths. The significance of absorption-line quasars has also been enhanced. We therefore undertook a study using today's greater set of data.

Table 1 Linear Polarisation of quasars, all Z_{em} values

		Received wavelength (cm)				
		3.7	6	11	31	49
Absorption lines	Number	21	16	24	17	16
	Median	2.8%	3.4%	2.2%	1.9%	1.1%
	Mean	$4.55 \pm 0.99\%$	$5.30 \pm 1.21\%$	$3.81 \pm 0.75\%$	$2.11 \pm 0.49\%$	$1.22 \pm 0.26\%$
No Absorption lines	All	Number	110	115	187	117
		Median	2.6%	3.1%	2.7%	1.5%
		Mean	$3.01 \pm 0.22\%$	$3.87 \pm 0.27\%$	$3.17 \pm 0.16\%$	$2.04 \pm 0.16\%$
	Equivalent numbers	Difference	$-1.54 \pm 1.01\%$	$-1.43 \pm 1.24\%$	$-0.64 \pm 0.77\%$	$-0.07 \pm 0.52\%$
		Number	16	16	26	16
		Median	1.6%	4.0%	2.7%	1.7%
	Same Z_{em} values	Mean	$2.40 \pm 0.55\%$	$4.42 \pm 0.67\%$	$3.28 \pm 0.37\%$	$1.91 \pm 0.35\%$
		Difference	$-2.15 \pm 1.13\%$	$-0.88 \pm 1.38\%$	$-0.53 \pm 0.84\%$	$-0.20 \pm 0.60\%$
		Number	20	18	24	19
		Median	3.2%	4.6%	2.6%	1.0%
		Mean	$3.17 \pm 0.59\%$	$4.54 \pm 0.77\%$	$3.15 \pm 0.45\%$	$1.80 \pm 0.41\%$
		Difference	$-1.38 \pm 1.15\%$	$-0.76 \pm 1.43\%$	$-0.66 \pm 0.87\%$	$-0.31 \pm 0.64\%$

Table 2 Linear polarisation of quasars, $Z_{em} > 1.25$

		Received wavelength (cm)				
		3.7	6	11	31	49
Absorption lines	Number	16	11	18	12	10
	Median	2.8%	3.4%	1.9%	1.9%	1.1%
	Mean	$4.25 \pm 1.14\%$	$5.44 \pm 1.60\%$	$3.52 \pm 0.85\%$	$1.62 \pm 0.38\%$	$0.95 \pm 0.27\%$
No absorption lines	Number	37	31	57	30	17
	Median	2.8%	3.6%	2.3%	1.7%	0.9%
	Mean	$3.03 \pm 0.39\%$	$4.29 \pm 0.57\%$	$3.01 \pm 0.29\%$	$2.01 \pm 0.29\%$	$1.35 \pm 0.33\%$
		Difference	$-1.22 \pm 1.20\%$	$-1.15 \pm 1.70\%$	$-0.51 \pm 0.90\%$	$0.39 \pm 0.48\%$

Measurements of linear polarisation at various radio wavelengths were taken from refs 3–10. Where several measurements existed for a given source at one wavelength, an average value was used. To be considered a quasar, a source had to be in the catalogue of Burbidge *et al.*¹¹, which also served as a reference for redshifts.

We calculated the mean and the median linear polarisation for various types of quasars at wavelengths $\lambda\lambda$ 3.7, 6, 11, 31, and 49 cm. Results are summarised in Tables 1 and 2. Table 1 includes all quasars regardless of redshift; Table 2 only those with $z_{em} > 1.25$, the redshift criterion of the earlier studies^{1,2}. The basic division is between those quasars having, and those not having, absorption lines. In Table 1 only, the latter are subdivided into three groups: (1) all quasars with non-absorption spectra; (2) the first 26 of them, in order of right ascension, constituting a sample comparable in size to that for the absorption-line quasars; and (3) a set consisting of quasars selected by emission redshift to match the emission redshift of an absorption-line mate. (The absorption-line quasars OQ172 and OH471, both with $Z_{em} > 3$, could not be matched better than $|\Delta Z_{em}| \sim 0.8$.)

In no case do we find a difference in mean linear polarisation between absorption and non-absorption quasars as large as two standard deviations. Only at λ 3.7 cm do we find differences in means even slightly larger than their associated errors, and these usually in the sense that absorption-line quasars have higher mean polarisations. We conclude that the linear polarisation properties of quasars having absorption lines are indistinguishable from those not having absorption lines.

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G. A. SEIELSTAD

Owens Valley Radio Observatory,
California Institute of Technology,
Pasadena, California 91125

Received 16 September; accepted 7 October 1977.

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Saturn-like ring system around Uranus

OBSERVATIONS made at Kavalur^{1,2} of the occultation of SAO158687 by Uranus on 10 March 1977 showed, in addition to the occultation by a satellite system termed the ϵ ring, a dimming of the light of the star close to the time when the atmosphere of the planet could have caused the phenomenon. Since then, the identities of the α , β , γ , δ rings have also become well established, and a theoretical interpretation of their origin³ has been proposed. We have closely examined the Kavalur photoelectric record and describe here several new features of the satellite ring system of Uranus.

Our analysis consisted of scaling the photoelectric record at close intervals to study the variation in intensity. The experimental details have been described elsewhere. After careful examination we have also picked out many large spikes not reported previously which show the nature of distribution of the occulting material in the vicinity of the planet. The scaling interval is 3 s, an order of magnitude greater than the time resolution of the recording system. After correcting for atmospheric extinction and a small linear drift in the recording system, the scaled values were grouped together to give a moving average over a time interval of 15 s. Figure 1 shows these values over the entire recording period, a striking feature is the significant reduction of light of the star during the intervals 2014 to 2031, 2032 to 2041, 2042 to 2055 and 2057 to 2106 UT. We term these zones A, B, C and D respectively. An additional zone of light loss occurs during 2108 to 2120 UT.

We interpret these zones A, B, C, D as caused by occulting material distributed in an extended region around the planet.

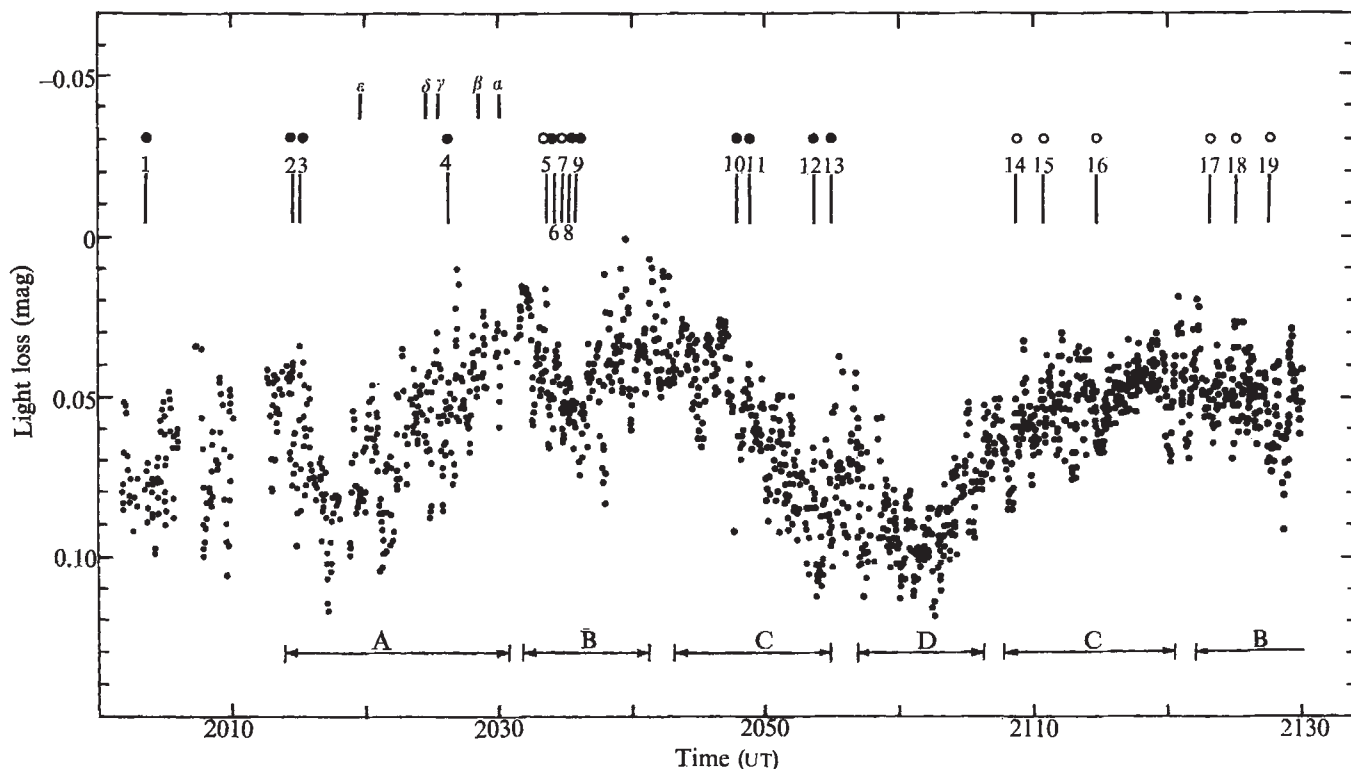


Fig. 1 Observed light variations at Kavalur of SAO158687 during 2000–2130 UT 10 March 1977. At the top the locations in the time coordinate where the ϵ , δ , γ , β and α rings intercept are indicated. A second row of indices numbered 1 to 19 are new spikes of short duration that we have identified on our record. The open and filled circles associated with these represent the degree of certainty in the identification of these spikes; the filled circles denote spike amplitudes that have a confidence level greater than 99.5%, while the open circles indicate visible spikes that are less certain.

Sharp discontinuities segregate these zones. A model for such a distribution of material is shown in Fig. 2 with extended ring systems A, B, C, D, similar to the ring system one sees in Saturn. Besides the occultation path, the α , β , γ , δ , ϵ ring condensations, the gaps in the particle distribution in the rings that appear as discontinuities, and the spike locations defining the new ring condensations that are listed in Table 1 are also marked. The distances are tentative and are on the measured basis of almanac positions of Uranus together with corrections for the relative positions of Uranus and SAO158687 according to Franz, Wasser-

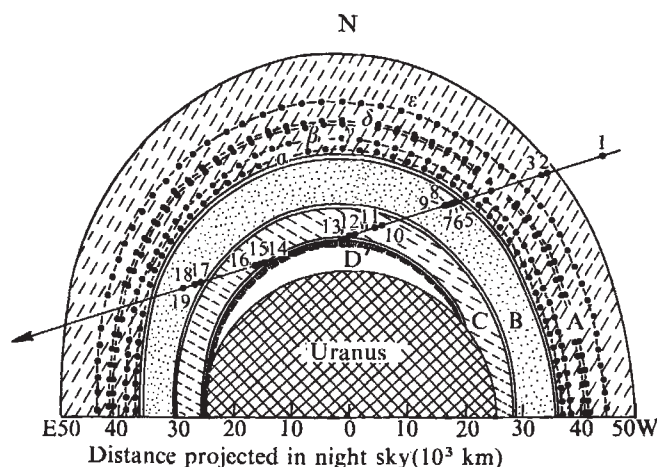
man and Seidelmann⁴. There is also the possibility of an inclination of the plane of the ring system to that of the equatorial plane of Uranus.

The C and D rings cause the light diminution reported earlier^{1,2}. The D-ring effects are maximum at 2102 UT. This ring is separated from the C ring by a wide gap of $\sim 2,000$ km. The two occasions when the light of the star encounters this gap are 2056 and 2107 UT. On the first intercept there is a near-restoration in brightness. The second event at 2107 UT is less striking. The lower boundary of the D ring is unspecified; Fig. 2 shows that it could extend nearer the planetary limb. There is considerable fine structure in both the C and D rings, as over the entire light curve of Fig. 1, portraying the light variations of the entire ring system.

Rings B and A are further away from the planet. The starlight encountered the opacity of the B ring material between 2032 and 2041 in the pre-occultation phase and again after 2122 UT. The B ring is well defined in Fig. 1, by a light loss of almost five hundredths of a magnitude. Our observations end when the light of the star is still affected by this ring on the post-occultation side. But the trend towards a reduction in intensity of starlight is clear from the light curve and substantiates our interpretation. We also note the discontinuity at 2041 and 2122 UT as caused by the gap between the B and the C rings. The light of SAO158687 shone through the A ring earlier than 2031 UT. The A–B gap is short at this epoch. The outer boundary of the A ring is poorly defined, partly because of lack of continuous record and partly because of the low altitude of Uranus at that time. Ring A contains the ring-condensations α , β , γ , δ and ϵ . There is obscuring material even beyond the boundary we have assigned to ring A as evidenced by the spike labelled 1 in Table 1.

The ring-condensations shown by the spikes on the record are distributed over the three outer rings A, B, C. We present in Table 1 the observed times of 19 such events together with their confidence levels. In the neighbourhood of each spike over an 80-s

Fig. 2 Diagrammatic representation of the Saturn-like ring system around Uranus. Dotted line indicates the lower boundary of the D ring.



interval of the record, we have evaluated the amplitudes of both positive and negative spikes that constitute the random noise. Assuming that this is gaussian we calculate the spike amplitude in terms of the standard deviation and read off from tables the confidence level for the spikes being of non-atmospheric origin. In Figs 1 and 2 we have listed only those spikes that are easily seen on the tracing; the lowest level of confidence of those in Table 1 is 97.9%.

These ring-condensations seem to vary in opacity along the ring plane. As examples, we cite the spike numbers 5 and 6 which coincide with events 4 and 5 reported by Millis, Wasserman and Birch⁵. Both of these events have very high confidence levels of 99.67 and 99.99% respectively on the Lowell records. (We determined this value from samples of Lowell tracing provided by Wasserman.) On the other hand, these events, on our records,

Table 1 Confidence levels for spike events

Event no.	Confidence level (%)	Event no.	Confidence level (%)
1	99.8	11	99.8
2	99.8	12	99.8
3	99.6	13	99.5
4	99.9	14	99.0
5	98.8	15	99.0
6	99.6	16	98.9
7	99.0	17	97.9
8	99.5	18	98.1
9	99.5	19	99.2
10	99.9		

have confidence levels of 98.8 and 99.6% respectively. In fact we record spikes of higher probability of occurrence than event 5 of Millis *et al.* The inference is, therefore, that longitudinal variations of opacity in the plane of the ring-condensations exist.

An exception seems to be the relatively wide ring-condensation termed ϵ . In Fig. 3 we give a light curve of occultation by this ring

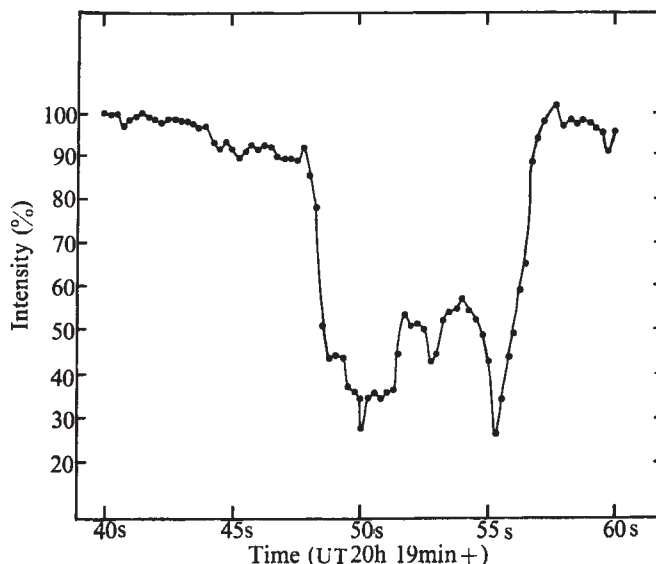


Fig. 3 Intensity variations caused by the ϵ ring.

from closely spaced measures of the photoelectric tracing every quarter of a second. Note that there is much fine structure in the variation of transparency that may be due to resonances associated with Titania and Oberon as postulated by Dermott and Gold³. Also of note is the close agreement between the light curve of the ring found by Millis *et al.*⁵ and by us, even though the intercepts with the ring are made at locations several thousand kilometres apart.

Table 2 summarises the above discussion of an extended ring system about Uranus, similar to that of Saturn. By dividing the Table into the pre- and post-occultation phases and locating events by time, we avoid the uncertainties of values of distance. If most of these particles are small and abundant enough to have

Table 2 The extended ring system of Uranus

Ring feature	Time of Crossing		Spike no.	Pre-occultation		Spike no.	Post-occultation	
	Pre-occultation	Post-occultation		Time (h min s)	Time (h min s)		Time (h min s)	Time (h min s)
Outer boundary	20 14		1	20 03 28				
ϵ ring-condensation	20 19 51		2	20 14 37				
δ ring-condensation	20 24 41		3	20 15 10				
γ ring-condensation	20 25 41							
β ring-condensation	20 28 40		4	20 26 21				
α ring-condensation	20 30 11							
Inner boundary	20 31							
Gap A-B								
Outer boundary	20 32		5	20 33 40				
B			6	20 34 08				
			7	20 34 47	19		21 27 37	
			8	20 35 15	18		21 25 08	
			9	20 35 48	17		21 23 12	
Inner boundary	20 41	21 22						
Gap B-C								
Outer boundary	20 42	21 20	10	20 48 06				
C			11	20 48 51	16		21 16 58	
			12	00 52 52	15		21 10 40	
			13	20 54 53	14		21 08 45	
Inner boundary	20 55	21 08						
Gap C-D								
D Outer boundary	20 57	21 06						

All times in UT

caused these opacity effects, their albedo, assuming them to be similar in constitution to those in Saturn's rings, should make the presence of the extended ring system observable.

J. C. BHATTACHARYYA
M. K. V. BAPPU

Indian Institute of Astrophysics,
Bangalore 560034,
India

Received 3 August; accepted 3 October 1977.

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Why is a minor planet minor?

ALL planets and satellites are generally believed to have formed by accretion of small bodies. The craters on the Moon, Mercury and Mars give clear evidence that the accretion of small bodies was of major importance, at least during the last phase of their formation. It is very likely that collision and fragmentation of planetesimals played a key part in the accretionary stage of planets. As suggested by Hartung¹, accumulation and fragmentation of planetesimals may have competed in the planetary accretion process, and a planetesimal which could survive catastrophic destruction may have become a planet. On the basis of the accretion model of planet formation, we propose a new idea in which we explain why minor planets are 'minor' and could not grow to a full-size planet. These results also substantiate Orowan's² idea that terrestrial planets accreted inhomogeneously, with iron being the first to accumulate and silicates the second.

Modes of fragmentation and accumulation of planetesimals during a collision depend on material property, impact velocity, and relative size of the impacting planetesimals. To simplify the discussion we consider only a mass ratio of projectile:target of 10^{-2} . If the target is the largest planetesimal in the system and the size distribution of the planetesimals follow $dn \propto m^{-5/3} dm$, then the planetesimals larger than the projectile of the above mass ratio comprise about 50% of the total mass. Our numerical simulation of planetary accretion³ also indicates that the collision of the planetesimals with above projectile:target mass ratio may control the evolution of the total planetesimals.

As a result of collision between two planetesimals, there are three possible fates: (1) bouncing, (2) accumulation, and (3) fragmentation or net mass loss of the target planetesimal. If the collision of two planetesimals is elastic, the two planetesimals will rebound from each other and the two will not coagulate, when the impact velocity is larger than the escape velocity of the target planetesimals. If the collision is made at a very large velocity, then the two planetesimals are broken into smaller fragments and major parts of the fragments may acquire kinetic energy large enough to escape from the two-body system. If the impact velocity is between the first (elastic) case and the second (fragmentation) case, ejecta material at the impact does not have a velocity large enough to escape from the target and it may eventually accumulate on the target planetesimals. Only this case will contribute to the net growth of the planetesimal.

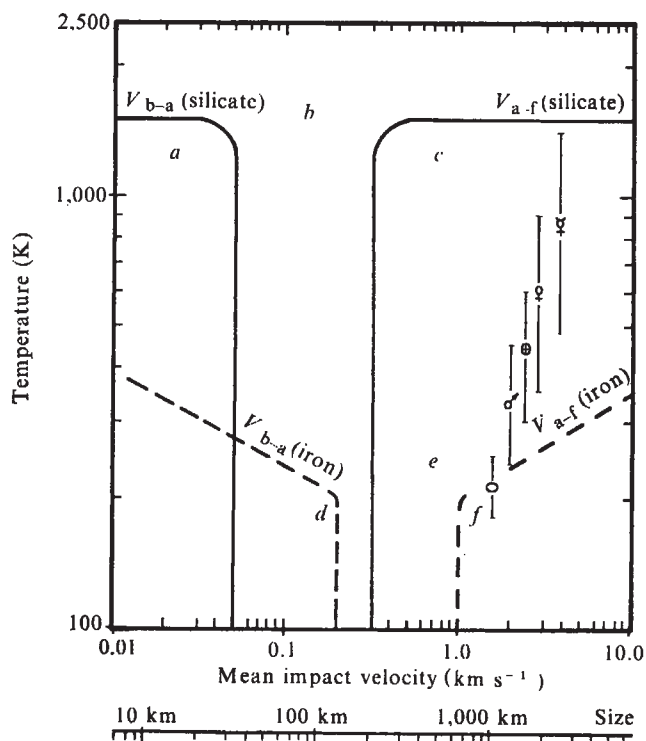
The critical impact velocity subdividing the above three cases depends on the material properties of the target and projectiles. For instance, if colliding bodies are very plastic, they are not easily disrupted but coagulate even at a rather high impact velocity. Orowan² first pointed out the important effect of the plastic (or ductile) property of iron on the planetary accretion process. He suggested that iron and

nickel are nucleating agents of the inner planets, because metallic particles are expected to stick together by 'cold welding' or 'hot welding' more easily than silicates.

It is important to note here that mechanical properties also change significantly with temperature. Iron is no longer ductile at temperatures below ~ 200 K, as shown by an impact experiment with the Gibeon iron meteorite⁴. Many other metallurgical studies⁵ also show that most irons undergo brittle-ductile transition at temperatures between 200 and 250 K. Therefore iron particles cannot be the nucleating agents at temperatures below 200–250 K. We propose here that this important factor determines the asteroid's evolution. Silicates become ductile only at a high temperature, however (for example, most silicate rocks would probably show the brittle-ductile transition at $T \sim 1,500$ K at impact conditions of interest).

The critical velocity, V_{a-f} , subdividing the accumulation regime and fragmentation regime, is estimated as follows. According to Gault and Wedekind⁶ and Fujiwara *et al.*⁷ a kinetic energy of 10^7 erg g^{-1} will be enough to completely fragment a basaltic body. When the mass ratio of the projectile:target is 10^{-2} , the velocity corresponding to the above energy is about 0.3 km s^{-1} ; V_{a-f} (silicate) ~ 0.3 km s^{-1} . In the case of an iron target at brittle condition, V_{a-f} is 5 to 10 times larger than that for a silicate rock, because iron has a higher tensile strength and a higher failure (strain) limit; both of these being a factor of 2 or 3 higher than ordinary silicate rocks. Therefore the critical velocity, V_{a-f} (iron) may be around 1 km s^{-1} at a brittle (low temperature) condition. On the other hand, at a ductile condition, $T > 200$ K for irons, $T > 1,500$ K for silicate rocks,

Fig. 1 Temperature and impact velocity conditions for bouncing, accumulation and fragmentation of planetesimals. The solid lines indicate boundaries for silicate material. The broken lines indicate boundaries for iron planetesimals. The symbols are for Mercury, Venus, Earth, Mars and Minor planets and show the estimated temperatures and mean impact velocities of planetesimals in each proto-planetary particulate ring. The scale of size corresponding to the escape velocity ($\sim$ the mean impact velocity here) is shown. *a*, Stone rebound; *b*, stone accrete; *c*, stone disrupt; *d*, metal rebound; *e* metal accrete; *f*, metal disrupt.



these materials are tougher than at a brittle condition, because most of the impact energy is absorbed for the plastic deformation of the material. Correspondingly, the critical velocity V_{a-f} at a ductile condition may become substantially higher than the above estimates. We expect, therefore, the boundaries dividing the accumulation and fragmentation regimes to be like those shown in Fig. 1.

The critical velocity, V_{b-a} , subdividing the bouncing and accumulation regimes is the minimum impact velocity which generates a stress higher than the elastic (tensile) limit, σ_e , of the material. A simple calculation yields the following values: assuming σ_e (silicate) = 0.5 kb, and σ_e (iron) = 5 kb, V_{b-a} (silicate) $\sim 0.05 \text{ km s}^{-1}$, and V_{b-a} (iron) $\sim 0.2 \text{ km s}^{-1}$. These estimates are consistent with impact experiments by Hartmann⁸ for silicate rocks. The critical velocity V_{b-a} also changes with temperature, because the strength of the material changes with the temperature. The velocity is expected to become smaller as the temperature increases.

Thus we obtain a map of the bouncing accumulation, and fragmentation regimes as a function of temperature and impact velocity, shown in Fig. 1. We stress that Fig. 1 is only schematic, because the exact position of the boundaries might be affected by the shape, impact angles and other properties of the colliding planetesimals.

In Fig. 1, silicate planetesimals can grow only in the region *b*, and iron planetesimals in region *e*. Therefore, only the area superposed by both the regions *b* and *e* satisfies the condition for both iron and silicate planetesimals to grow. In other regions only one component of them (either iron or silicate) or neither of them, can grow.

The next problem is to estimate temperatures and mean impact velocities for planetesimals in a proto-planetary particulate disk. According to Lewis⁹, the bulk compositions of Mercury, Venus, Earth, Mars and the asteroids Ceres and Vesta are dominated by materials formed in the condensation temperatures around 1,400 K, 900 K, 600 K, 450 K and 250 K respectively. These temperatures may give upper bounds of planetesimal temperatures in each planet zone. Lower bounds on the planetesimal temperatures are probably given by the radiative-equilibrium temperature at the present planet positions. These are 480 K, 350 K, 300 K, 240 K and 180 K, for Mercury, Venus, Mars and the asteroids respectively. We tentatively assume that the temperature of planetesimals during the accretion-collision stage lies between these two limits.

It is also very difficult to estimate the impact velocities. If there are no dense gases in the terrestrial planet's region at the time of accretion, the planetesimal may acquire a relatively high velocity due to the distant and/or close encounters of other planetesimals. The mean relative velocity of planetesimals may be approximated by $V^2 = (e^2 + i^2) v_k^2$, where e , i , and v_k are eccentricity, inclination and Kepler orbital velocity of each planet. If we take the mean eccentricity and inclination of the terrestrial planets ($e = 0.08$ and $i = 0.0$), the mean velocity of planetesimals in the terrestrial planet region becomes higher than 1 km s^{-1} .

The temperatures and the mean impact velocities estimated above are plotted in Fig. 1. As seen, almost all the data points lie in the region (*c+e*), where only iron-like materials can coagulate. This observation supports Orowan's idea that iron-like material is the nucleating agent in the terrestrial planets. Silicates could probably not accumulate until the iron-nucleus grew to about 1,000 km in radius. This result gives another dynamical mechanism for heterogeneous accretion of planets.

Figure 1 indicates that asteroids are in a marginal position; the asteroid condition may fall in region *f*. This indicates that iron-like planetesimals in the asteroid zone had difficulty in growing due to their brittleness at low temperature, so that iron could not be a nucleating agent in the asteroid zone. Probably the iron-like material could

not grow at all or would grow too slowly to become large enough to form a planetary nucleus. The small iron planetesimal also fails to capture enough silicate material for a full-size planet. Thus, it is very likely that the low temperature condition of the asteroids hampered their growth into iron-like planetesimals, which in turn inhibited the accumulation of silicate planetesimals. In conclusion, we propose that the brittleness of iron at temperatures below 200–250 K is the primary reason why minor planets are minor, and why minor planets could not grow to full-size planets.

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TAKAFUMI MATSUI

The Lunar Science Institute,
3303 NASA Road 1,
Houston, Texas 77058

HITOSHI MIZUTANI

Geophysical Institute,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

Received 27 August; accepted 7 October 1977.

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Kimberlites and plate-tectonics in West Africa

MESOZOIC kimberlites in West Africa are the source of most of the alluvial deposits of diamond which bolster the economies of Sierra Leone, Guinea, Liberia, Ivory Coast and Ghana. Difficulties encountered in the interpretation of geophysical, geochemical and mineralogical prospecting methods led to a structural investigation into the distribution and tectonic controls of kimberlite magmatism in eastern Sierra Leone. Here we attempt to show a correlation between the continental continuations of transform fault fracture zones and kimberlite magmatism in West Africa.

Areas of richly diamondiferous alluvial deposits, unabraded picro-ilmenite and pyrope occurrences and outcrops of kimberlite pipes and dykes indicate a broad east–north-east swathe of kimberlitic activity cutting through West Africa from Sierra Leone to northern Ghana^{1–4}. Figure 1 shows that Mesozoic kimberlite fields within this zone of activity are frequently centred near well developed north–south brittle structures, as in eastern Sierra Leone and western Ivory Coast. In Sierra Leone, east–north-east trending kimberlite dykes cut across Archaean north–south and south-east trending structures, but parallel local east–north-east fractures confined between major north–south regional faults. Only in the coastal strip of Sierra Leone and Liberia are regional scale east–north-east structures apparent; elsewhere, they are short, disconnected structures of only local importance.

The Atlantic Ocean floor is cut by still active transform fault fracture zones which are orientated along east–north-east directions where they intersect with the coastline^{5,6}. Two such fracture zones intersect the Sierra Leone coastline; the Guinea Fracture Zone and the Sierra Leone Fracture Zone. When traced inland their hypothetical continuations are the loci of kimberlitic intrusions, but are not major structures as in Nigeria⁷, being subtly incorporated into the regional structure. The Sierra Leone Frac-

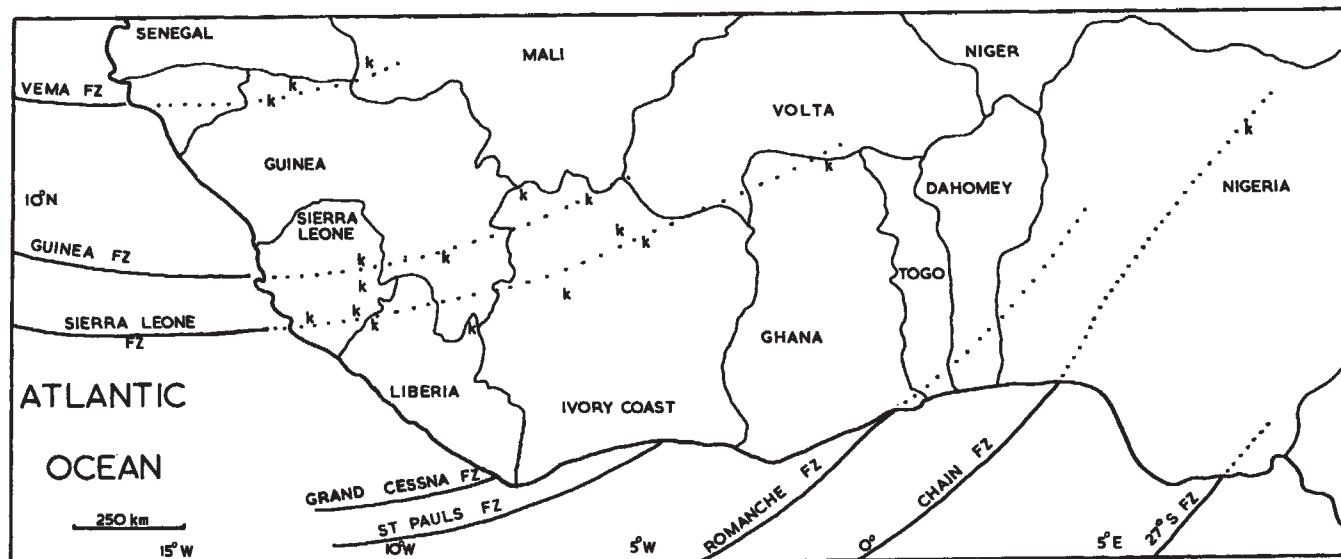


Fig. 1 West Africa, showing locations of kimberlites of probable Mesozoic age, (k); oceanic fracture zones (FZ) and their hypothetical continental continuations.

ture Zone may be traced 150 km inland as the fault-controlled estuary and course of the Sewa River, while the Guinea Fracture Zone meets the coastline at Freetown, and is recognisable as a major fault-controlled estuary along which two layered basic intrusions are developed⁸.

Mesozoic kimberlitic activity occurs elsewhere in West Africa, in northern Guinea and in Nigeria⁷. In Nigeria, kimberlite is found, as in Sierra Leone, close to the continental continuation of a major fracture zone, the Chain. In Mali⁴, the close association of kimberlites and Mesozoic basalts suggests a Mesozoic age for the former. In northern Guinea and Mali, the kimberlites lie on the hypothetical continental continuation of the Vema Fracture Zone. Similar relationships between magmatism and oceanic fracture zones were proposed by Marsh⁹. He supported the hypothesis that the continental expressions of the fracture zones were small circles about the pole of rotation of seafloor spreading. In West Africa, the pre-80 Myr spreading took place about a pole situated at 21.5°N, 14°W (ref. 10). At the time kimberlites were being intruded into the crust, the pole of rotation for the Africa-America spreading system was moving north towards its present position¹¹. During pole migration the fracture zones and their continental expressions became activated, perhaps allowing increased penetration of kimberlitic magma into the crust. The absence of kimberlites along hypothetical continental extensions of fracture zones such as the St Pauls and Romanche may be due simply to the lack of kimberlitic magma along these structures, or to insufficient tectonism to allow its upward movement.

A relationship between kimberlites and structures associated with plate-tectonics is suggested in West Africa, and confirms a hypothesis based on south-west African data⁹.

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H. R. WILLIAMS
R. A. WILLIAMS

Department of Geology,
University of Sierra Leone,
Freetown,
Sierra Leone

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Laminar-turbulent transition in polymer solutions

WHETHER transition from laminar to turbulent flow is delayed in dilute drag-reducing polymer solutions, unchanged, or occurs at an earlier Reynold's number has been a vexing question since research began in this area. Using the classic Reynold's dye-streak technique, Giles and Pettit¹ showed that pipe-flow turbulence was delayed by a factor of 2 or more by small additions of polymer. Similarly, Castro and Squire² and White and McEligot³ found transition delay in small pipes from measurements of pressure fluctuations and actual drag-reduction performance. But more refined pipe-flow studies by Little *et al.*⁴ show convincing evidence for polymer solution transition earlier than for the pure solvent and this has been confirmed by laser-Doppler anemometry⁵. Here we show that polymer solutions in a boundary-layer flow exhibit a transition to turbulence at a lower Reynold's number than the pure water solvent.

Following the work of Brennen⁶, an ogival-shaped head with an axial length of 3½ inches and a base diameter of 2.34 inches was tested in the Naval Academy free-surface water tunnel. The head shape corresponds to a source distribution, cut off sharply and supported by a long sting through which air was supplied to ventilate the cavity, and through which the cavity pressure could also be measured. By ventilation, a fully developed cavity could be supported at a Reynold's number (based on ogive length) of 3×10^3 and a cavitation number, σ , of 0.5 where $\sigma = (P_T - P_v) / \frac{1}{2} \rho U^2$ and P_T and P_v are the tunnel and cavity pressures and U is the tunnel velocity (10 ft s⁻¹). The ventilated cavity as seen by the eye is shown in Fig. 1a; the cavity rising with respect to the water flow due to its buoyancy.

When the cavity is illuminated by a stroboscope, or photographed using a short-duration flash, the series of waves shown in Fig. 1b is readily discernible on the cavity in pure-water flow. These waves represent the cavity-flow

equivalent to the instabilities in a free shear layer in a (one-phase) wake flow and have been studied by Brennen⁶. Note that the flow leaving the ogive surface is laminar, and the waves are part of the laminar-turbulent transition process on the surface of the cavity.

At the same tunnel velocity and pressures, but with 25 p.p.m. solution of polyacrylamide (Calgon TRO-375) instead of water, the laminar zone in the flow leaving the ogive has disappeared as shown in Fig. 1c. A similar appearance to Fig. 1c was obtained by roughening the surface of the ogive in pure water flow (although we were careful to avoid roughening the ogive surface when using polymer solution). Thus, we conclude that the presence of polymer additives causes an earlier transition to turbulence in the boundary layer of the ogive than does the pure-water flow. A similar conclusion could be drawn from photographs of water and polymer solution jets discharging in air⁷, but the test geometry is not as well defined as in the present study.

At the wall shear rate involved (10^4 s^{-1}), the presence of the polymer has increased the water viscosity in the tunnel by about 40% (as estimated from rheograms for a polymer of similar drag-reduction effectiveness⁸) and thus the Reynolds number for the polymer flow has decreased to around 2×10^5 . The effect of the polymer on transition is, hence, even more marked than indicated by the photographs.

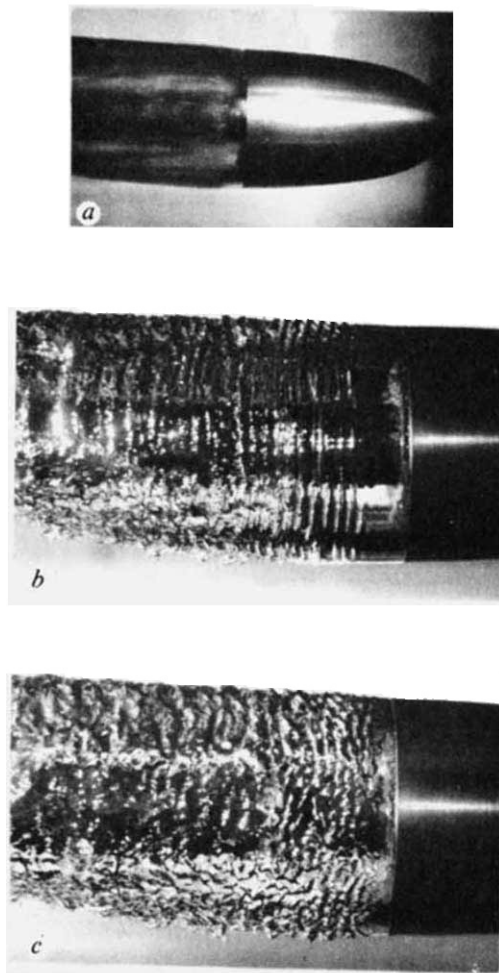


Fig. 1 Photographs of ogive head-form in water tunnel. (Flow is from right to left.) a, Overall view of body and cavity; b, short-duration flash (10 μs) showing laminar flow leaving ogive and transition to turbulence. $R_0 = 3 \times 10^5$, $\sigma = 0.5$; c, same tunnel velocity and pressures as b but 25 p.p.m. polyacrylamide solution instead of pure water. $R_0 = 2 \times 10^5$, $\sigma = 0.5$.

The demonstration in polymer solution flow of a destabilising effect on transition, together with the known diminishing of turbulence production at higher Reynolds numbers should have an important influence on the development of theoretical explanations of the mechanism of polymer drag reduction.

J. W. HOYT

Naval Systems Engineering Department,
US Naval Academy,
Annapolis, Maryland 21402

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Time sharing and body partitioning in bat-plant pollination systems

IN the dry months from January to March in Central America there is an abundance of flowering trees and shrubs¹⁻³ and many of them are pollinated by bats. When collecting nectar, the bats visit various species of plants⁴⁻⁶, and in periods of peak abundance of flowers they make increasingly frequent visits. With flowering apparently synchronised^{3,4}, there seems to be a good chance that flowers may not always receive conspecific pollen, thus reducing their seed set and fitness. The study reported here suggests that this chance is minimised because nectar and pollen are available at different times on different flower species, and because some species deposit and receive pollen at distinct regions of the bats' bodies.

Interspecific competition among plants sharing common elements of the pollinator fauna is seen in entomophilous systems when weeds or native plants prove superior to crop plants^{7,8}. The disadvantage of being a minority species in such a community free-for-all has been shown by Levin and Anderson⁹—given a finite number of visits by pollinators, sharing of pollinators by plant species might decrease the chances of appropriate pollination for less numerous species, resulting in lowered seed set and eventual elimination from the community. Thus, a hypothetical case can be made for mixed species associations becoming increasingly oligotypic. But real tropical communities persist in polytypy, which shows that plants avoid this dilemma.

Sympatric insect-pollinated plants with similar phenologies seldom interbreed because they: (1) are incompatible; (2) 'use' relatively specific pollinators; (3) deposit pollen on different parts of the pollinator's body, and (4) present pollen and/or nectar rewards at different times^{10,11}. I suggest that time sharing and body partitioning are 'escapes' from competition available to some bat-adapted plants.

The feeding habits of bats have been investigated previously by stomach and faecal analysis^{5,6,12}, and by swabbing the fur with an adhesive substance and then examining the swabbed material microscopically^{4,13}. Workers using such pooled pollen analyses have interpreted mixed pollen loads to indicate lack of discrimination by the bats. For example, Heithaus *et al.*⁴ found six species of pollen in 42% of 527 loads examined and concluded that: "The high proportion of mixed pollen loads . . . suggests low flower constancy in bats". But the methods used in all these studies did not or could not take into account the distribution of pollen temporally or topographically. For plants to have the benefits of pollinator specificity, bats need not feed only on one species. Effectiveness of pollination would be enhanced if bats confined each of their several nightly visits for feeding to a single species, or if they visited different species on different nights or at different times.

While working in Costa Rica and British Honduras in March 1974, 1975 and 1976, I netted bats in several sites between 1930

Table 1 Pollen grains from fur of *Glossophaga soricina*, March 1974 and 1975

Central farm, British Honduras <i>N</i> = 26							
Time	<i>Bauhinia</i> (large-small)	<i>Inga</i>	<i>Roupala</i>	<i>Ipomoea</i>	<i>Calliandra</i>	<i>Caryocar</i>	Bombacaceae
1900-2100		1,600	200				
2200-0200				557	10		
0200-0400	1,267	12		1			123
0400-0600		2	1				
Santa Rosa, Costa Rica <i>N</i> = 22							
1900-2100		500				220	
2000-0200							913
0200-0400	870	3					640
0400-0800	300	17					2

and 0530 h. I swabbed pollen from the heads, shoulders, venters and rumps of glossophagine bats, making separate slide preparations for each anatomical region and noting the times of capture. Table 1 shows that *Glossophaga soricina* Pallas concentrates on *Inga* (Mimosaceae) early in the evening and switches to *Bauhinia* (Caesalpinaceae) towards dawn.

The way in which plants may reinforce or entrain such behaviour is shown in Fig. 1; nightly nectar flow is timed correspondingly. *Inga* anthers are ripe between 2000 and 2100 h, whereas *Bauhinia* anthers do not dehisce until the early morning (0400-0600 h). This suggests that the availability of pollen is also co-adapted with bat schedules. The visual acuity of nectar bats is fairly good¹⁴, and the well developed olfactory lobes and vomeronasal organ¹⁵⁻¹⁷ indicate that olfaction plays a large role in their orientation. Flower discrimination and concomitant time-entrainment should be no more difficult for bats than for bees. As Darwin pointed out¹⁸, such constancy affords the pollinator more efficient foraging as well as facilitating appropriate pollination. Further bat and plant studies in other months or in different sites may reveal more of this trend to time partitioning.

While netting along a transect from Monte Verde, Guanacaste, Costa Rica to a point 5 km west, I captured eight female and four male *Anoura geoffroyi* Grey. Two yielded pure *Mucuna* (Papilionaceae) pollen, probably *M. urens*, placed ventrally. The other 10 carried mixed loads, *Mucuna* on the venter, *Crescentia* (Bignoniaceae) on the shoulders and mid-dorsum and pure *Inga* (six bats) or mixed *Inga* and unidentified Bombacaceae (four bats) on the face and neck. Essentially the plants were using three different and fairly constant pollinators.

These data do not preclude overlap in pollinator 'use' by many plants. For example, Alvarez and Gonzalez Quintero⁵ found mixed pollen loads on *Glossophaga* and *Anoura*: four or five genera were often well represented. Although the methods used (stomach and faecal analysis) may have hidden a microtemporal

structure which could be revealed by further work, it is unlikely that all genera found in pooled pollen samples were involved in time sharing.

Another example is provided by *Bauhinia unguolata*, *Bombacopsis quinata*, *Ceiba pentandra*, *Luchea speciosa* and *Pseudobombax septenatum*, which can bloom synchronously in the same site¹⁹. All these bat-adapted species have morphological adaptations to deposit pollen on the head, suggesting that floral homologues 'use' identical pollinators. As Baker²⁰ indicated, taxonomically unrelated flowers may well be able to share a pollinator where the supply of that animal is adequate. Only when pollinators are scarce would such behaviour on the part of the plants become disadvantageous. At such times sympatric species which partition pollinators may be able to co-exist more successfully than those that do not²¹. Competition resulting from a low density of pollinators would then reinforce floral differences or promote such differentiation, reinforcing and maintaining community polytypy.

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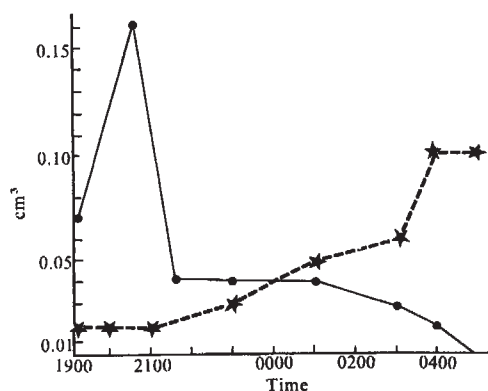
D. J. HOWELL

Biology Department,
Purdue University,
West Lafayette,
Indiana 47907

Received 12 July; accepted 28 September 1977.

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Fig. 1 Nightly nectar flow in two bat-pollinated species. Nectar was extracted with a 1-cm³ syringe and blunt needle. Points for each species represent averages of 10 flowers from two trees. ●—●, *Inga marginata*; — — —, *Bauhinia unguolata*.



Possible case of aggressive mimicry in a neotropical scale-eating fish

Probolodus heterostomus, one of the scale-eating characoid fishes, is found in the coastal rivers of southeastern Brazil from Espírito Santo to São Paulo¹⁻³. The species is usually placed in the subfamily Cheirodontinae²⁻⁴ although a taxonomic affinity with the Tetragonopterinae has been suggested¹. The latter view was apparently based largely on the morphological similarity of *Probolodus* with some species of *Astyanax* and especially *A. fasciatus*, a member of the

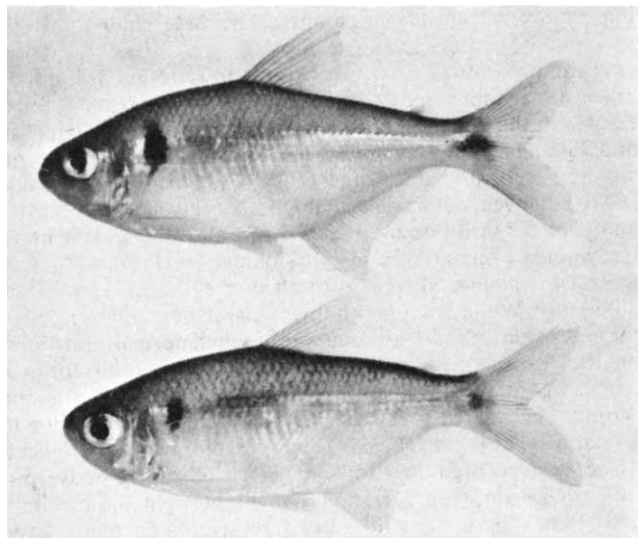


Fig. 1 *P. heterostomus* (upper) and *A. fasciatus* (lower) from the Rio Paraibuna, São Paulo. The colour of both fishes is also very similar; actual size of *Probolodus* 76 mm.

Tetragonopterinae. The external similarity between the two species, which are sympatric over the Rio Paraíba drainage, is indeed striking (Fig. 1). But data accumulated from population samples, stomach contents and the behaviour of individuals maintained in aquaria suggest that *Astyanax* is the principal prey of *Probolodus*, at least in certain areas of its range. I suggest here that the similarity between these two genera is a form of aggressive mimicry^{5,6} irrespective of taxonomic affinities.

Thirty-five individuals of *P. heterostomus* (54–104 mm) were caught in the Rio Paraibuna, São Paulo (22°23'S, 45°40'W), on five occasions between May and July 1977. The fish were collected by means of casting net, bow-net and hook. On the same occasions a general collection of other fishes was also made. On all occasions *Probolodus* was caught together with *A. fasciatus* (total 35 individuals compared with 409) which strongly suggests that these two species shoal together or at least live in close proximity. On three occasions, 24 individuals of the curimatid *Curimatus gilberti* were also caught together with *Probolodus* and *Astyanax*. Other scale-bearing fishes found sympatrically with *Probolodus* were the erythrinid *Hoplias* (one species); the characids *Astyanax* (two additional species), *Hyphessobrycon* (one species) and *Oligosarcus* (one species); the anostomid *Leporinus* (two species); and the cichlids *Geophagus* (one species), *Cichla* (one species) and the introduced *Tilapia* (one species). For these, no comparable numerical data were obtained but they were far less numerous than *A. fasciatus* and only incidentally collected together with it and *Probolodus*.

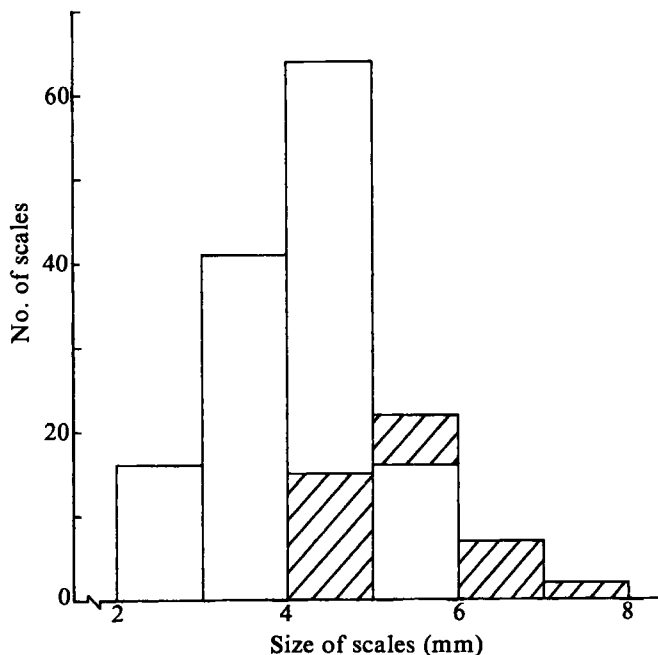
With the exception of four individuals which were maintained alive all *Probolodus* were killed immediately and preserved. These preserved specimens were scored for length and their stomach contents were analysed. Relatively undigested scales from 31 stomachs were stained with alizarin red and compared with a reference collection of scales from the fishes of the general collection (Fig. 2). In this sample 183 scales were clearly recognised as from *Astyanax* and *Curimatus*. It was not possible to identify with certainty the remaining, more digested scales, but only eight were of the ctenoid type (attributable to *Tilapia*) while all others, about 600, were cycloid of the *Astyanax* and *Curimatus* type. Other food items included plant material and insects.

The four live fish (64–82 mm) were maintained separately in aquaria (50–160 l) and used in observations of feeding behaviour. In one series of observations two to four individuals of *Astyanax* (36–115 mm) were placed together with

each *Probolodus*, and the groups were observed in sessions of 15–40 min for about 40 h. Soon after the introduction of *Astyanax*, *Probolodus* shoaled with them, and at intervals fed on their scales. In the most frequently observed feeding strategy *Probolodus* followed closely an individual of *Astyanax*, manoeuvred itself obliquely to the rear of the victim and struck at its flanks, securing one to a few scales on each successful attack. The scales seemed to be removed merely by a gaping and closing of the mouth during the strike, sometimes accompanied by a quick lateral movement of the head. The scales were swallowed immediately and *Probolodus* resumed shoaling. During the first day or so *Astyanax* shoaled together with *Probolodus* and apparently failed to recognise it as a 'predator'. Subsequently, *Astyanax* avoided close proximity with *Probolodus* and, to a certain extent, with each other. This latter behaviour is perhaps the result of repeated attacks on the same individuals in the confinement of the aquarium. In natural conditions the possibility of recognising *Probolodus* as a predator would be diminished because attacks would be 'diluted' over a greater number of victims. Fish less than half the size of *Probolodus* individuals, or more than one and a half times as large, were unmolested, which suggests that prey of more or less the same size as the predator are preferred.

In other observations, two individuals of diverse species were introduced separately into the aquarium with a *Probolodus* individual. These species included the cichlid *Geophagus brasiliensis* (60–81 mm), the characid *Bryconamericus* sp. (50–55 mm), the cyprinid *Carassius auratus* (41–53 mm) and the poeciliid *Phalloceros caudimaculatus* (22–32 mm). After initial attacks both *Bryconamericus* and *Carassius* avoided the close presence of *Probolodus*, hid and needed to be pursued. Only the largest *Probolodus* (82 mm) attacked *Geophagus*, and to a much lesser extent than the other species, while the small *Phalloceros* went unmolested. The latter observation confirms the size preference shown previously by *Probolodus*. In a final series of observations, both *Astyanax* (two individuals) and *Geophagus* (one individual) were introduced into an aquarium with *Probolodus*.

Fig. 2 Number and size of 183 relatively undigested scales from stomach analyses of 31 individuals of *P. heterostomus*. Size extremes for each genus may be found in the same individual prey and also in the stomach of the same predator. Open columns, *Astyanax*; hatched columns, *Curimatus*.



After 24 h *Astyanax* had suffered much greater scale loss than *Geophagus*.

Taken together, the accumulated data provide support for the hypothesis of aggressive mimicry^{5,6} in which *Astyanax* is the prey-model and *Probolodus* is the predator-mimic. First, *P. heterostomus* and *A. fasciatus* have a striking overall similarity to each other. Aquarium observations indicate that *Astyanax* tolerated *Probolodus* and failed to recognise it as a potential predator, at least until repeated scale-biting in the conditions of a small aquarium caused a break-down of the shoaling habit. Second, both species are sympatric in the Rio Paraibuna (and over the Rio Paraíba drainage) and apparently live in mixed shoals or in close proximity. The proportion of *Probolodus* individuals to *Astyanax* in these areas seems to be roughly 1:10 which again fits the mimicry requirements. Third, *Probolodus* is a specialised scale-eater with a preference for prey more or less its own size and which is closely followed and struck from the rear. Stomach analyses of individuals caught in the Rio Paraibuna show preponderance of *Astyanax* scales.

A comparable case of mimicry has been reported for the African scale-eating cichlid fish *Corematodus shiranus* which closely resembles and shoals with the cichlid species *Tilapia squamipinnis*^{7,8}. In such cases, the mimicry of the predator apparently facilitates its predatory success by allowing it to maintain close proximity with its potential prey⁵⁻⁹. This is the classic tactic of the wolf in sheep's clothing. Even if *Probolodus* is not so selective as it seems from my data it may still be advantageous to have the appearance of an ubiquitous and harmless fish, to facilitate the approach to an intended victim. A comparable strategy is used by a bird of prey¹⁰ and some marine fishes^{11,12}.

As *A. fasciatus* is not found over the entire range of *Probolodus*, it is probable that in other major river drainages this latter fish associates with other similar species of *Astyanax* (in the Rio Ribeira do Iguape, *A. ribeirae* is a likely candidate) or even such tetragonopterines as *Deuterodon iguape* which also has the general appearance of *Astyanax*.

Drs P. E. Gibbs, E. O. Willis and P. Montouchet reviewed the manuscript and Dr H. E. Britski confirmed the identity of the fishes.

IVAN SAZIMA

Departamento de Zoologia,
Universidade Estadual de Campinas,
13100 Campinas, São Paulo, Brazil

Received 30 August; accepted 6 October 1977.

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A new insect chemosterilant isolated from *Acorus calamus* L.

PLANT extracts have been tested for insecticide and juvenile hormone¹ activity and some have been found with anti-juvenile hormone activity². We have suggested^{3,4} that an essential oil of *Acorus calamus* L. inhibits interstitial cell activity; this would represent a new concept in insect chemosterilisation. We report here the identification of the component of the oil that is responsible for that inhibition; we have also established the

importance of substitute groups and a side chain by testing various allyl benzene analogues.

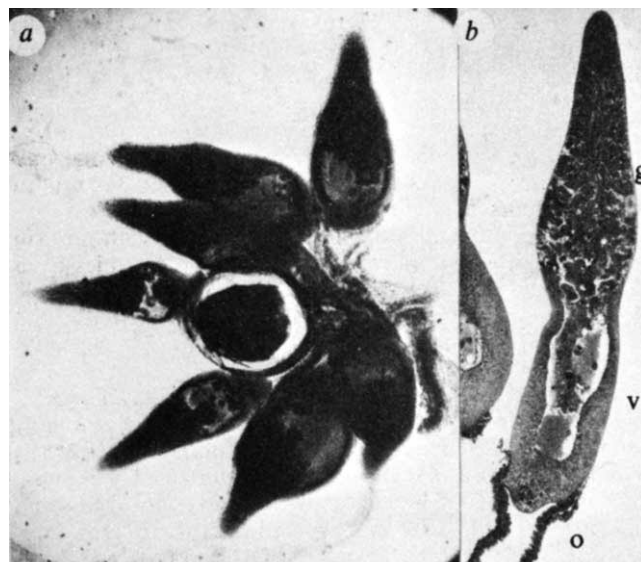
Using procedures described before^{3,4}, we tested 3rd, 4th and 5th instar nymphs and the adults of *Dysdercus koenigii* for the efficacy of the inhibitory agent. Victoria blue⁵, aldehyde fuchsin⁶ and Sudan black B were used to test for the appearance of neuro-secretory cells and interstitial cells (IC). Farnsyl methyl ether (FME), a juvenile hormone analogue, was given topically (10 µg and 12 µg per insect) to the insects before and after treatment with the candidate oil to reveal any physiological reversal.

Acorus calamus oil seemed to affect the various larval stages differently. While 3rd and 4th instar larvae moulted normally to the next instars, the 5th instar larvae moulted normally into adults but the ovaries were irreversibly affected. In such adults the ovary remains permanently immature (Fig. 1a). Longitudinal section through such an ovary revealed a well developed tropharium but a small vitellar region (Fig. 1b), composed only of interfollicular tissue, and no organised follicles. Isolation of the active principle⁷, β -asarone (Fig. 2, 1) showed that it brought about a similar inhibition. The compound caused resorption in many gravid insects leading ultimately to complete regression of the ovaries. To find which of the substituent groups in the aromatic ring or the side chain of β -asarone was specifically inhibitory, trials were done with different allyl benzene compounds (Fig. 2). A wide spectrum of activity was observed (Table 1).

Table 1 shows that compound 1 severely depressed the development of the gonads. The neuro-secretory cells (NSC) revealed by both Victoria blue and aldehyde fuchsin were scored according to Schooneveld⁸ and Johnson *et al.*⁹. The studies revealed no inhibition in the NSC's and the density of granules was nearly equal to that in controls. In cases where higher dosages (above 30 µl per 1,500 cm³ of air space) were tried, however, a mild inhibition in the secretion from NSC was observed. There is no reason to suppose that NSC activity is involved in the chemosterilant action of β -asarone, because it has been reported that extirpation of the NSC within 30 h of the moult to adult does not affect subsequent vitellogenesis in *D. cingulatus*¹⁰.

Because the treated insects showed a normal moulting pattern we conclude that the oil does not adversely affect ecdysone release. As previously shown¹¹, on treatment with higher doses, the moulting insect becomes lethally entangled in its own half cast moult. This may be because the larger dose of oil inhibits the release of secretion by NSC, which then delays the release of ecdysone and thus seriously impedes ecdysis.

Fig. 1 a, Ovary of 6-d-old adult treated with *Acorus* oil. Note the well developed germarium and oocytes as spherical bodies in vitellar cavity. $\times 15$. b, Longitudinal section through the ovariole of above ovary. Note the well developed tropharium (g) a short vitellarium (v) and oviduct (o) $\times 75$.



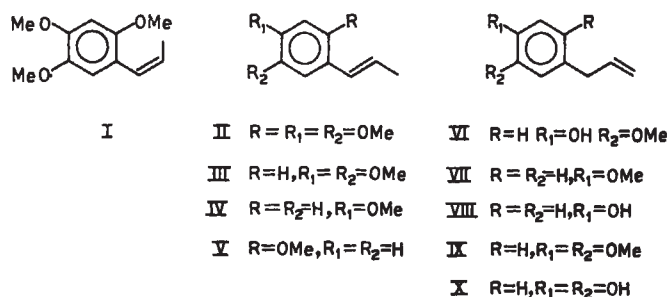


Fig. 2 Structural formulae of compounds used.

The anti-juvenile hormones precocene I and II possess a significant measure of structural similarity with β -asarone, and both types of compound cause sterility in adult female insects. But they probably have different modes of action. Thus precocenes are believed to act by blocking the synthesis and release of juvenile hormones, which leads to the formation of precocious adults when the larvae of sensitive species are treated². By contrast, β -asarone did not cause precocious metamorphosis in *D. koenigii* in our experiments. Furthermore, we observed no physiological reversal of the β -asarone effect on insect gonads when FME was applied topically to the insects before or after β -asarone treatment. These observations were made on adults up to 7d after moulting, which coincides with the first oviposition of control insects.

The molecular structure of the effective compound from oil of *A. calamus* is given in Fig. 2 I. The position of different groups in the aromatic ring is known to play an important part in various activities, as shown by some allelochemicals¹², where the position of the hydroxyl groups is responsible for activities such as anti-assimilation and reduction of survival or pupation. Similarly in our study the position of the *O*-methyl groups on the ring, and of the double bond in the side-chain of β -asarone, plays an important part in the antagonistic activity (Table 1, Fig. 2). After testing the various compounds it was found that the *O*-methyl group at position R in the ring (Fig. 2, V) is important for the resorption of the oocytes, which is further supported by the suppression in the ovary due to the *O*-methyl group at R₂ (as in Eugenol, VI and methyl isoeugenol, III—Fig. 2). The suppression in the ovary due to the *O*-methyl group at R₂ seems true of precocene II² also because the removal of the *O*-methyl group from R₂ position in that molecule also reduces activity (precocene I of Bowers²). Comparison of the activities of compounds I and II (Table 1) suggests that the *cis* configuration of the side-chain double bond in β -asarone is important for antagonistic activity. It is interesting, however, that III which has a *trans* configuration, but lacks the *O*-methyl group at position R also has significant suppression activity.

Table 1 Activities of test compounds

Compounds	Activity		
	10 μ l per 1,500 cm ³ air space	20 μ l per 1,500 cm ³ air space	30 μ l per 1,500 cm ³ air space
I	Antigonadal	Antigonadal	Antigonadal
II	Nil	Nil	Nil
III	Nil	Mild yolk deposition*	Antiaassimilant + no yolk deposition
IV	Nil	Toxic	Toxic
V	Nil	Nil	Antigonadal
VI	Nil	Antiaassimilant + no yolk deposition	Toxic
VII	Nil	Nil	Nil
VIII	Nil	Nil	Nil
IX	Antifeedant	Toxic	Toxic
X	Nil	Nil	Nil

An antagonistic compound sterilised either sex, agglutinated sperm and caused resorption from terminal oocytes to apex of vitellarium. In antiaassimilants the alimentary canal was full and the fat body highly reduced and in antifeedants the alimentary canal was always empty.

*Mild yolk deposition: the effect was the same in all oocytes.

We conclude that β -asarone is neither a juvenile hormone, nor an anti-allatotrophic compound (precocene I and II of Bowers²), and with its specific antagonistic functions, it may represent a new type of antagonistic agent which may afford a new and safe approach towards insect control.

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BHASKAR P. SAXENA
OPENDER KOUL
K. TIKKU
C. K. ATAL

Regional Research
Laboratory (CSIR)
Jammu Tawi—180001,
India

Received 14 June; accepted 12 October 1977.

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Assignment of aberration breakpoints in banded chromosomes

CHROMOSOME banding techniques^{1–4} have made possible not only the detection but also a more accurate characterisation of the structural chromosomal changes encountered in clinical and experimental work. Using internationally agreed band patterns and nomenclature⁵, it is customary to assign breakpoints to aberrations such as translocations on the assumption that these 'breakpoints' correspond to the positions of breakage and rejoining which gave rise to the configuration observed. This assumption is frequently invalid, because it does not seem to be generally realised that the system carries (at least for chromosome-type exchanges) an inherent three-band uncertainty. This means that the most obvious breakpoint is not necessarily the site of transfer or rearrangement of the genetic material.

The assignments of 'breakpoints' is essentially an exercise in pattern recognition. Presented with a linear array of alternating dark and light segments against a light background (for example, a typical G-banded chromosome set viewed with conventional light microscopy), the human eye automatically concentrates on the disposition of the dark bands. We are much more conscious of differences in size, spacing and staining intensity of dark bands than of light bands although such differences occur in the latter.

Interruptions or breaks in an expected sequence are therefore interpreted in terms of the dark band pattern, and the pattern disruption point (PDP) will be assigned to a light band. Figure 1 illustrates this for a 6:10 human reciprocal translocation using the Paris Conference (1971) diagrams and nomenclature⁵. The left-hand pairs represent the G band patterns of the normal and derivative chromosomes. Obvious pattern disruption occurs at 6q21 and 10q22, that is a light/light-band exchange. But, essentially the same pattern (and derivative chromosome lengths and arm ratios) could be achieved by dark/dark-band exchanges on either side of this light-band, that is 6q16/10q21 or 6q22/10q23, but because of the eye's preoccupation with dark-bands, this is not immediately obvious.

This three-band uncertainty becomes clear when the same translocation is R banded⁶, a technique whereby the Giemsa staining of the bands is reversed in a more or less reciprocal manner (Fig. 1, right-hand pairs). The eye transfers attention to the new

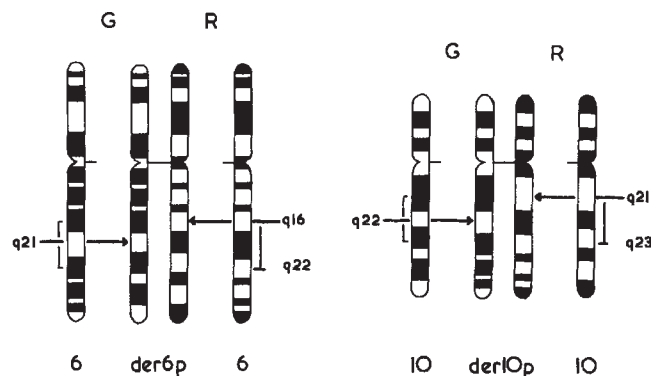


Fig. 1 The derivative chromosomes of a human 6:10 reciprocal translocation compared with normal homologues. Left-hand pairs, G-band pattern; right-hand pairs, R-band pattern. Arrows indicate the pattern disruption points (PDP), and the three-band uncertainty zone is bracketed.

dark-band sequence so that the PDP again falls in a light band. This corresponds to one or other of the dark G bands which make up the trio of uncertainty. The breakpoint seems to have jumped a band, but only as the result of an optical artefact; it is not necessary to infer that exchange at a band junction is involved⁷. In the example of Fig. 1, the choice of PDP depends on the assessment of the R dark bands 6q21 and 10q22. Relative size differences are obvious in the diagram, and careful measurement will probably implicate 6q21 and 10q22 as the most likely breakpoint positions. In the real cell, such differences are seldom obvious, as we are working near the limits of optical resolution, and the quality of the banding is rarely uniform throughout the chromosome complement, so that homologue differences are often present.

In clinical work, where a particular translocation can be examined by different techniques and in several cells, subtle differences in size, staining intensity and conformation, impossible to depict in a diagram, may allow assignment of the breakpoint with reasonable certainty, but not in every case. In experimental work, particularly with primary chromosome-type changes, usually only one example is available, and the three-band uncertainty frequently remains. Where uncertainty exists it may be safer to use a more non-committal term like PDP.

It is often said that most chromosome-type exchange breakpoints occur in G light-bands (or weak fluorescing bands which are mostly identical to light bands)⁸⁻¹¹. This could be largely an artefact of pattern-recognition.

Non-exchange aberrations such as simple terminal deletions do not, however, have the three-band uncertainty. This is also the case for most chromatid-type aberrations, because sister-chromatid pairing affinity ensures a banded 'control' chromatid in close proximity to the exchange point. Thus band-for-band comparisons can be made¹²⁻¹⁴. When this is done for X-ray-induced exchanges there is a clear indication of more or less exclusive light/light band involvement.

Free participation of both dark and light bands should lead to ~ 50% dark/light-band exchanges, which would be recognised by the creation of an extra dark band in one of the derivative chromosomes. Such cases seem to be very rare, implying that either exchanges are predominantly light/light or dark/dark or that partial bands are unstable and tend to merge with bands adjacent to the point of exchange. A similar argument applies to exchanges involving band junctions, interfaces¹⁵, or 'interbands'⁷. A high frequency of free interface/interface exchange would produce frequent band fusion in one derivative and concomitant band loss in the other. Such events are seldom seen.

Terms like band 'interface' and 'interband' should be used with caution. Sharp dark/light band junctions only exist on diagrams. The metaphase chromatid is a coiled¹⁶ or double-coiled¹⁷ structure, and there have been several suggestions that bands and coils are related¹⁸⁻²⁰. Thus a transverse line across a chromatid will

run more or less parallel to the pitch angle of the coil, and is therefore unlikely to represent a sharp boundary for breakage purposes.

JOHN R. K. SAVAGE

MRC Radiobiology Unit,
Harwell, Didcot,
Oxon, UK

Received 26 July; accepted 10 October 1977.

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***Tetrahymena pyriformis* recovers from antibody immobilisation by producing univalent antibody fragments**

MANY protozoa possess mechanisms by which they are able to recover from immobilisation by specific antibodies. Paramecia have been shown to shed their ciliar antigens in response to specific divalent antibodies. After shedding the antibody bound antigens the animals present new antigens of different specificity¹. It has been suggested that this modulation process also has a role in antigen variation in trypanosomes². While modulation of serotypes in *Tetrahymena pyriformis* is primarily provoked by temperature shifts or changes in culture conditions³, induction of modulation in response to antibody agglutination has been reported^{4,5}. *Tetrahymena* recovery from antibody provoked immobilisation has also been reported to occur by a nonspecific process not involving a change in serotype. Immobilised animals have been shown to secrete a substance which permits their recovery and protects animals of unrelated serotype from homologous anti-serum⁶⁻⁸. We have further examined the way that *Tetrahymena* recover from antibody provoked immobilisation. This process, which we call fabulation, apparently involves the proteolytic cleavage of both bound and free immunoglobulin to produce univalent fragments which bind or remain bound to the ciliar antigens and protect the animals from further antibody treatment with no apparent effects on cell growth. The cleavage seems to be catalysed by both cell bound and secreted proteases.

When *Tetrahymena* of syngen 9 are treated with specific antisera they are rapidly immobilised and often agglutinated. Rabbit antisera to *T. pyriformis* were prepared either by the method described in ref. 4 or by two intradermal injections of 5×10^5 animals in complete Freund's adjuvant at 6-week intervals, followed by two intravenous injections of 10^4 sonicated animals at 2-week intervals. Rabbits were bled 6 d after the last injection. Immobilisation and agglutination tests were performed as described in ref. 4, except that the cell concentration was 2–4,000 per ml.

We have observed that when immobilised animals are left at room temperature for 1–2 h in the presence of antiserum all the animals are found to have recovered and swim freely. Further treatment of the recovered animals with antiserum of the same specificity has no effect. Also, addition of antisera directed to other serotypes which the animals are capable of expressing has no effect. The possibility that the recovered animals had modulated their serotype and presented a hitherto unknown antigen was tested by injecting the recovered animals into rabbits. We found, however,

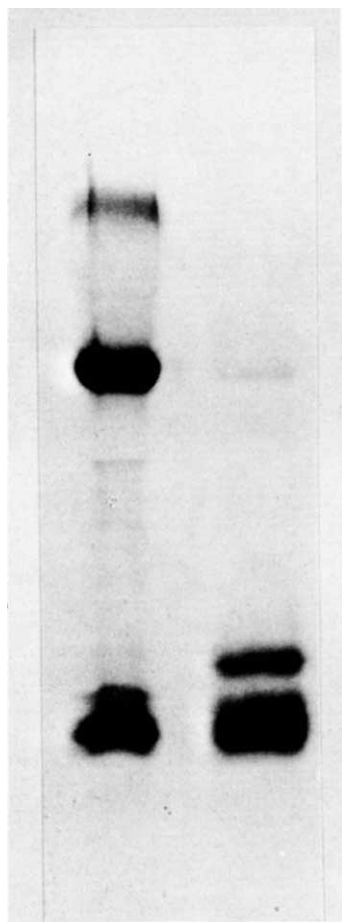


Fig. 1 Effect of *Tetrahymena* cells on ^{14}C -labelled mouse immunoglobulin. ^{14}C -labelled mouse immunoglobulin, prepared by incubating spleen cells from trypanosome infected mice in a mixture of ^{14}C amino acids, was a gift from Dr B. Bernstein. 10^4 *Tetrahymena* grown as described in ref. 6 were incubated with $5\ \mu\text{l}$ of ^{14}C -immunoglobulin (12,000 c.p.m.) in 10^{-2}M phosphate buffer pH 7.5 containing 10^{-3}M EDTA and 10^{-3}M dithiothreitol. After 1 h at 37°C , 2 volumes of $2\times$ sample¹² buffer were added and the samples were boiled for 2 min. They were then analysed by electrophoresis on 10% polyacrylamide gels as described by Laemmli¹³. The gels were dried and autoradiographed. The left lane represents the immunoglobulin preparation incubated without *Tetrahymena* cells. The right lane represents the preparation incubated with the cells.

that this resulted only in the production of very weak sera against the original serotype. It thus seems unlikely that the animals recover by a process of modulation of their ciliar antigens. If the recovered animals are cultivated in antiserum free medium (containing normal rabbit serum in the place of anti-*Tetrahymena* rabbit serum) they become sensitive to the original antiserum within 2–3 generations. The animals can also be rendered sensitive to the original antiserum by exhaustive washing. It seems, therefore, that the recovered animals are protected from the action of further antibodies.

Tetrahymena contain and excrete several proteases amongst which is an enzyme resembling papain^{9,10}. As papain is known to cleave IgG of various mammalian species to produce Fab fragments¹¹, it seemed possible that the *Tetrahymena* recover from antiserum treatment by specific cleavage of the antibodies by the papain-like enzyme and production of Fab-like fragments capable of binding to the antigens. These bound univalent fragments would then protect the animals without apparently deranging them. If such a mechanism were responsible for the recovery of *Tetrahymena* from antibody agglutination it would be expected that the recovered animals should be sensitive to agglutination by antibodies specific for the immunoglobulin originally used to agglutinate them and from which they had recovered. Furthermore, antibodies specific for the non-antigen binding fragment, or Fc, would not be expected to have an effect on the recovered animals. To test these predictions animals were treated with

specific rabbit anti-*Tetrahymena* serum and allowed to recover. The recovered animals were then washed once and treated with either sheep anti-rabbit total IgG (SaRIgG) or with sheep anti-rabbit Fc (SaRfC). It was seen that treatment of the recovered animals with SaRIgG resulted in re-agglutination whereas treatment with SaRfC had no effect. SaRIgG treatment of animals grown in pre-immune rabbit serum or in the absence of serum had no effect. Incubation of the reagglutinated animals again resulted in recovery, showing that sheep IgG is also sensitive to the *Tetrahymena* enzyme(s).

To demonstrate the cleavage of IgG by *Tetrahymena* ^{14}C -labelled mouse IgG was incubated at 37°C in the presence of 10^{-3}M EDTA and 10^{-3}M dithiothreitol with washed *Tetrahymena* organisms. This treatment did not affect the viability of the organisms. After incubation the samples were analysed by SDS polyacrylamide gel electrophoresis. The gel was then dried and autoradiographed. The results are shown in Fig. 1. It can be seen that the light chain of the IgG was not degraded by the cells. The heavy chain was almost completely lost and replaced by two closely spaced bands which migrated just behind the light chain. The preparation of ^{14}C -labelled mouse immunoglobulin also contained a small quantity of IgM. It can be seen that the μ chain also disappeared after treatment with the cells although it is not known whether Fab was produced from the IgM. Similar results are seen when the immunoglobulins are incubated with *Tetrahymena* culture supernatants.

It seems clear from these observations that *Tetrahymena* of syngen 9 can recover from antibody agglutination by cleavage of the antibodies to produce univalent fragments which remain bound to the antigens and protect the animals from the effects of further antibody treatment. This 'fabulation' process suggests an efficient mechanism by which parasitic protozoa might escape the host immune system. Cell-bound univalent antibodies would be expected to have two effects: they would block antigenic sites from antigen sensitive cells, thus reducing the host's immune response, and they would protect the parasites from circulating antibody. Furthermore, cell-bound Fab would not activate complement.

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H. EISEN*

Department of Molecular Biology,
University of Geneva, Geneva, Switzerland

I. TALLAN

Department of Zoology,
University of Toronto, Toronto, Canada

Received 22 April; accepted 7 October 1977.

*Present address: Department of Molecular Biology, Institut Pasteur, Paris, France.

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Isolation of a human teratoma cell line which expresses F9 antigen

THE F9 antigen, defined by antisera raised in syngeneic mice against pluripotent embryonal carcinoma cells, is present on early mouse embryos, spermatozoa and male germinal cells, but not on adult somatic tissues^{1,2}. This antigen, thought to be coded by gene(s) located at, or linked to, the developmentally important *T/t* complex, may play a part in early embryogenesis^{3–5}. This idea is supported by the fact that the antigen seems to have been conserved during mammalian evolution. Anti-F9 activity is absorbed by sperm of several

species including man, rat, rabbit and bull³⁻⁷ and there is evidence for its presence on the morulae of rabbit, rat and cow, and on human foetal testicular cells^{2,5}. The cross reaction of anti-F9 with human sperm suggested that undifferentiated stem cells in human teratocarcinomas might also carry F9 on their surface, and we report here the isolation of human teratocarcinoma cell line which expresses F9 antigen.

The cell line, known as SuSa, was isolated from a 30-yr-old male with a malignant testicular teratoma, intermediate grade B with extensive necrosis. Sections of the primary tumour had areas of undifferentiated cells in convoluted epithelial sheets, networks and vesicles, surrounded by simple tumour mesenchyme; limited areas of more differentiated cells, including keratinised and glandular epithelium and cartilage were also present. The patient had

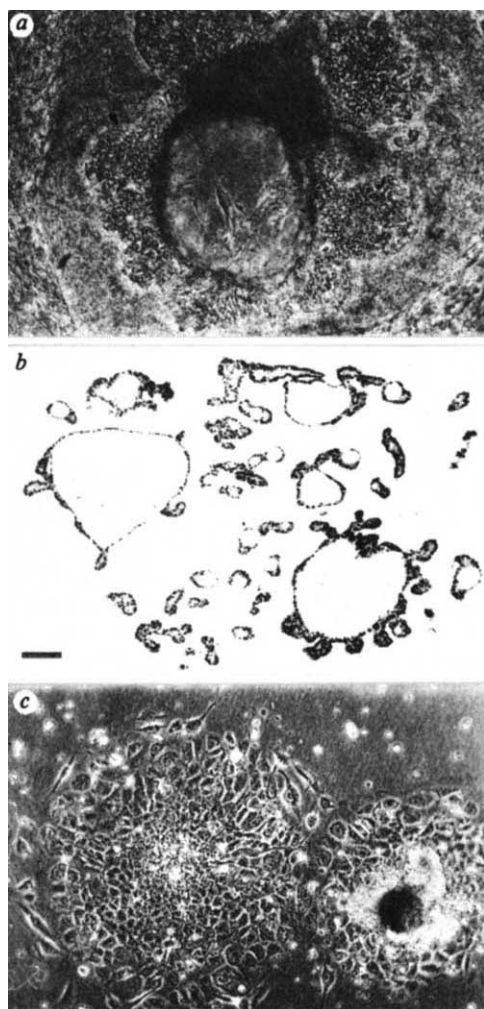


Fig. 1 Morphology of SuSa cells in culture. Pieces from different sites in the primary tumour were minced finely with scissors and placed in a 90-mm dish containing a monolayer of 3×10^6 STO mouse feeder fibroblasts⁸ in Dulbecco's modified Eagle's medium (MEM) with 10% calf and 10% foetal bovine serum. After about 5 d small patches of closely packed, homogeneous cells were seen, and after 2 weeks these were subcultured with 0.05% trypsin-0.5 mM EDTA in phosphate-buffered saline (PBS), pH 7.2. The cells have a very low plating efficiency (about 4%) even on feeder layers. Feeder layers were prepared by treating STO cells before plating out with mitomycin C ($20 \mu\text{g ml}^{-1}$) for 2-3 h and 6,000-10,000 rad X rays. *a*, A vesicle in the centre of a colony of cells growing at high density on a feeder layer. Phase contrast microscopy; bar represents 100 μm . *b*, Section through a chain of vesicles detached from a high density culture. Stained with haematoxylin and eosin, bar represents 50 μm . *c*, Cells growing out from a vesicle which had attached to a tissue culture dish in the absence of feeder fibroblasts; bar represents 100 μm .

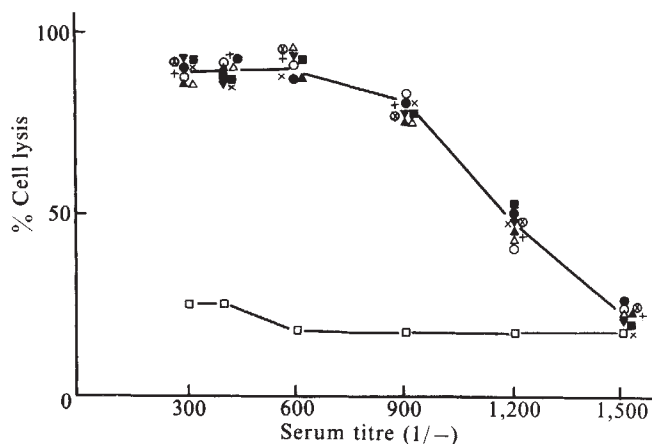


Fig. 2 Absorption of anti-F9 activity by SuSa and other human cell lines and mouse STO fibroblasts. Anti-F9 serum was elicited by hyperimmunisation of male 129/Sv mice with irradiated cells of the mouse teratocarcinoma line F9-41¹, and was absorbed before use with cells of the parietal yolk sac line PYS2 and with human fibroblast (skin) cells (v/v, serum diluted 1/5 in Hanks medium containing 4% heat-inactivated λ globulin-free foetal bovine serum (IPT), for 1 h at 0 °C). Such absorbed sera are active against F9 cells in both the microcytotoxicity test (90-95% of cells killed at titres of 1/2400-1/3200) and the indirect immunofluorescence assay (90-100% of the F9 cells labelled at dilutions up to 1/400). Absorptions were made with 100 μl of anti-F9 sera diluted 1/300 in Hanks medium+4% IPT. After absorption with 8×10^6 SuSa cells or 1×10^7 cells of all the other cell lines tested at 4 °C for 1 h, the cells were removed by centrifugation and the residual cytotoxic activity was tested on F9 cells using serial dilutions in the presence of absorbed rabbit serum as a source of complement¹. ●, Unabsorbed serum; □, absorbed on SuSa; △, absorbed on Burkitt's lymphoma⁹; ▲, absorbed on an intestinal cell line (American Type Culture Collection CCL6); ×, absorbed on an epidermal larynx tumour (ATCC CCL23); +, absorbed on HeLa (ATCC CCL2); ▼, absorbed on melanomas¹⁰; ⊗, absorbed on a rhabdomyosarcoma (ATCC CCL136); ○, absorbed on STO mouse fibroblasts.

multiple metastases and high levels of human chorionic-gonadotropin in the urine. HLA typing of the patient's peripheral blood lymphocytes was A1, A3, B5, Bw17 and possibly Cw3.

The cells are maintained on feeder layers of mouse fibroblasts and have a low ratio of cytoplasm to nucleus, and prominent nucleoli. They grow as flat colonies of densely packed cells which gradually pile up at the edges and centre, forming chains of simple epithelial vesicles which float off into the medium (Fig. 1a). Similar vesicles are produced when SuSa cells are subcultured in the absence of feeder fibroblasts. When floating vesicles attach to the tissue culture dish, the cells migrate out and become flattened (Fig. 1c), but they do not continue to grow when subcultured in the absence of feeder fibroblasts. In sections of fixed and stained material (Fig. 1b) SuSa cells closely resemble the undifferentiated epithelial cells of the primary tumour. In the electron microscope they show very little endoplasmic reticulum but have some microvilli and lipid droplets, and are joined by junction complexes. No virus particles have been seen.

Approximately 10 passages after its isolation the cell line had a modal chromosome number of 58, and its human origin was confirmed by the presence of metacentric chromosomes. Quinacrine mustard staining showed one Y chromosome in most spreads.

Absorption of anti-F9 serum with 8×10^6 SuSa cells removed cytotoxic activity towards F9 cells (Fig. 2). Further experiments showed that as few as 3×10^6 SuSa cells could remove most of the anti-F9 activity of the sera in the conditions described in Fig. 2. Anti-F9 activity was not absorbed by the STO cells used as feeders, nor by any of the human cell lines tested—HeLa, two Burkitt tumours,

two melanomas, an epidermal carcinoma of the larynx and an embryonal rhabdomyosarcoma.

In immunofluorescence studies (Table 1) anti-F9 serum reacted strongly with about half of the SuSa plus feeder cell population, but marked neither human nor mouse fibroblasts. No fluorescence labelling was observed after absorption of the serum by F9 cells or human sperm. Because mouse embryonal carcinoma cells which are F9 positive do not express H2 antigens or β_2 microglobulin⁵ we tested SuSa cells for the products of the major histocompatibility HLA locus in man. We found that only a very small percentage of SuSa cells was labelled by specific HLA-A1 or B5 antisera. The percentage of cells that bound anti-human β_2 microglobulin in serum correlated well with the percentage of HLA positive cells. Together with the absorption studies, these results show that cultured SuSa cells express an antigen which cross-reacts with the F9 antigen on mouse embryonal carcinoma cells and human sperm, and that this expression is not due to the presence of STO feeder cells. Furthermore, most cells expressing the F9 antigen do not express HLA antigens, and judging by the presence of HLA antigens, only a minority of the human cells in the cultures seem to be differentiated.

Table 2 shows the results of double immunofluorescence labelling. When anti-F9 and a specific anti-HLA-B5 serum were used simultaneously all F9 positive cells were HLA-B5 negative, and *vice versa*. The result was similar when a rabbit anti-HLA serum was used in combination with anti-F9, suggesting mutual exclusion of F9 and HLA antigens on the SuSa cells. Double labelling with anti-F9 and an anti-human β_2 microglobulin serum revealed that all F9 positive cells were β_2 microglobulin negative. The human nature of the F9 positive cells in the SuSa population was confirmed by

Table 1 Immunofluorescence studies on cell line SuSa, human fibroblast HLA-B5 and mouse fibroblast STO

Antisera used	% Cells fluorescing		
	Human teratoma SuSa	Human fibroblast HLA-A1 or HLA-B5	Mouse fibroblast STO
Anti-F9+ goat anti-mouse FITC	48,46,44,50	0	0
Anti-F9 preabsorbed on F9 + goat anti-mouse FITC	2	0	0
Anti-F9 preabsorbed on human sperm + goat anti-mouse FITC	4	0	0
Anti-HLA-B5 TRITC	6,5	80	0
Anti-HLA-A1 FITC	3,4	100	0
Rabbit anti-HLA+ goat anti-rabbit TRITC	8	100	0
Rabbit anti-human β_2 microglobulin + goat anti-rabbit TRITC	10,7	90	0

5×10^5 cells were centrifuged in a microfuge (Beckman Instruments Palo Alto). The pellet was resuspended in 50 μ l of antisera at an appropriate dilution in MEM+4% IPT and kept at 4°C. After 45 min the cells were washed with the same medium and suspended in an anti-IgG globulin labelled either with fluorescein isothiocyanate (FITC) (Hyland) or with tetramethyl rhodamine isothiocyanate (TRITC). After 45 min at 4°C, the cells were washed three times, resuspended in PBS pH 7.2, spread, air dried, fixed with methanol, then mounted in glycerol: PBS (80/20; v/v). Preparations were observed using a Leitz Orthoplan microscope. Appropriate specificity controls (normal and absorbed immune sera) were performed. Anti-F9 serum was used at a dilution of 1/80 and revealed by a goat-anti-mouse IgG and IgM, labelled with FITC. In the F9 controls, absorptions were made v/v with F9 antisera and cells for 1 h at 4°C. Anti-HLA-A1 and anti-HLA-B5 were obtained from multiparous women. Anti-HLA-A1 was labelled directly with FITC. Anti-HLA-B5 was labelled directly with TRITC. Both sera were used at 1/25 dilutions and had been absorbed on mouse STO cells before use. The rabbit anti-HLA 5996-2 and the rabbit anti human β_2 microglobulin sera (5895-21) raised against purified preparations of papain-solubilised HLA antigen and of human β_2 microglobulin were given by N. Tanigaki. They were both preabsorbed with the Daudi lymphoma and STO cells before use. They were revealed with goat anti-rabbit IgG labelled with TRITC.

Table 2 Double marking immunofluorescence studies on cell line SuSa

First antisera	Second antisera	% Positive cells
Anti-F9+ goat anti-mouse FITC	TRITC-anti-HLA-B5	48 F9 ⁺ HLA ⁻ , 0 F9 ⁺ HLA ⁺ 47 F9 ⁻ HLA ⁻ , 5 F9 ⁻ HLA ⁺
Anti-F9+ goat anti-mouse FITC	Rabbit anti-HLA+ goat anti-rabbit TRITC	44 F9 ⁺ HLA ⁻ , 0 F9 ⁺ HLA ⁺ 48 F9 ⁻ HLA ⁻ , 8 F9 ⁻ HLA ⁺
Anti-F9+ goat anti-mouse FITC	Rabbit anti-human β_2 microglobulin+ goat anti-rabbit TRITC	50 F9 ⁺ β_2 m ⁻ , 0 F9 ⁺ β_2 m ⁺ 40 F9 ⁻ β_2 m ⁻ , 10F9 ⁻ β_2 m ⁺
Anti-F9+ goat anti-mouse FITC	Rabbit anti-human fibroblast+ goat anti-rabbit TRITC	0 F9 ⁺ Man ⁻ , 32 F9 ⁺ Man ⁺ 24 F9 ⁻ Man ⁻ , 44 F9 ⁻ Man ⁺

See Table 1. Anti-human fibroblast serum was raised in rabbits immunised with three injections of human skin fibroblasts from a single donor. The serum was absorbed on mouse liver and spleen and on the mouse fibroblast line STO to render it specific. This serum is active on both HLA positive and HLA negative (Daudi) human cell lines. It was used at a dilution of 1/2,000 and revealed with goat anti-rabbit TRITC or FITC.

a double labelling experiment using anti-F9 and a pre-absorbed rabbit anti-human fibroblast serum. All F9 positive cells were labelled by this serum.

The studies described so far involved dissociated single cells. When intact aggregates were tested for F9 using methods applied to preimplantation embryos⁴, about 30% of them were marked by anti-F9 antiserum. When aggregates were dissociated before exposure to antiserum, 50–55% of the cells were F9 positive while about 80% were marked by anti-human fibroblast serum.

Several lines of evidence suggest that SuSa cells are equivalent to the undifferentiated stem cells of human testicular teratocarcinomas. First, most of them resemble morphologically the undifferentiated epithelial cells of the original tumour and other testicular teratomas^{11–13}. Second, most of them express an antigen which cross-reacts with the F9 antigen on mouse embryonal carcinoma cells, and third, they do not express, or have available for reaction, products of the major histocompatibility HLA locus or β_2 microglobulin. Although a few HLA positive cells are found in SuSa cultures we have no conclusive morphological evidence that the cells differentiate *in vitro*, nor can we exclude the possibility that the cells are differentiating into cytotrophoblast, which may not express HLA antigens. Proof of the tumorigenicity and stem cell nature of SuSa cells would be unambiguous if single cell clones gave rise to differentiated tumours in nude mice. But when eight nude mice were each injected subcutaneously with 1×10^6 SuSa cells, no tumours developed after 2 months. In earlier experiments, cells taken directly from five primary testicular teratomas and one ovarian teratoma were tested for F9 by absorption and found to be negative (Buc. G. Gachelin and M.F. unpublished results). This may have reflected the small proportion of undifferentiated stem cells in the tumours tested but we cannot formally exclude the possibility that SuSa cells have acquired F9 expression *in vitro*.

Our results and those of Holden *et al.* (see following communication)¹⁴ who find F9 on another line of human teratocarcinoma cells, Tera I, raise the possibility of looking for circulating autoantibodies in patients with testicular teratoma (and possibly seminoma), using cell lines such as SuSa, Tera I and F9 as targets.

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B. HOGAN

Imperial Cancer Research Fund,
Mill Hill Laboratories,
Burtonhole Lane,
London, NW7, UK

M. FELLOUS

Unité de Génétique Cellulaire de l'Institut Pasteur
et du Collège de France

P. AVNER

Department d'Immunologie et Virologie des Tumeurs,
U152, Hôpital Cochin, Rue du Faubourg St Jacques,
Paris, 75014, France

F. JACOB

Unité de Génétique Cellulaire de l'Institut Pasteur
et du Collège de France,
25 rue du Docteur Roux/Hôpital Saint Louis,
Paris, 75015, France

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Human and mouse embryonal carcinoma cells in culture share an embryonic antigen (F9)

THE embryonic membrane antigen F9 has been detected on several lines of mouse embryonal carcinoma (EC) cells as well as on mouse embryos and sperm, but not on any other adult cells tested¹. F9 or an antigenically similar substance has been demonstrated on the morula and/or sperm of several mammalian species² including man³, but has been undetectable in all non-mammalian species tested². Although no function for this antigen has been identified, its restriction to embryonic and germ cells suggests an important developmental role. This possibility is enhanced by the apparent conservation of F9 in the course of evolution. We now report the serological detection of F9 on human EC, together with our inability to detect β_2 microglobulin.

We investigated two established tissue culture lines of human EC cells for the presence of F9 antigen. These lines (Tera I, Tera II) were cultured from the pulmonary metastases of two men with testicular teratocarcinoma⁴. *In vitro*, these EC cells seem morphologically similar to murine EC cells and do not exhibit visible differentiation in standard growth conditions. Attempts are in progress to characterise these lines by growing them in athymic mice. Using mouse anti-F9 antisera, we have shown in complement-mediated cytotoxicity assays and by absorption that F9 antigen is detectable on Tera II but not on Tera I (Figs 1 and 2). F9 could not be detected by similar techniques on normal human fibroblasts or on three human lines derived from lung, intestinal and skin tumours. It is interesting that in direct cytotoxicity tests, Tera II was almost as sensitive to anti-F9 antisera as its murine counterpart.

Immunoperoxidase studies confirmed the presence of F9 antigen on Tera II cells and also showed that F9 can be detected on a small fraction of the Tera I cell population (Fig. 3).

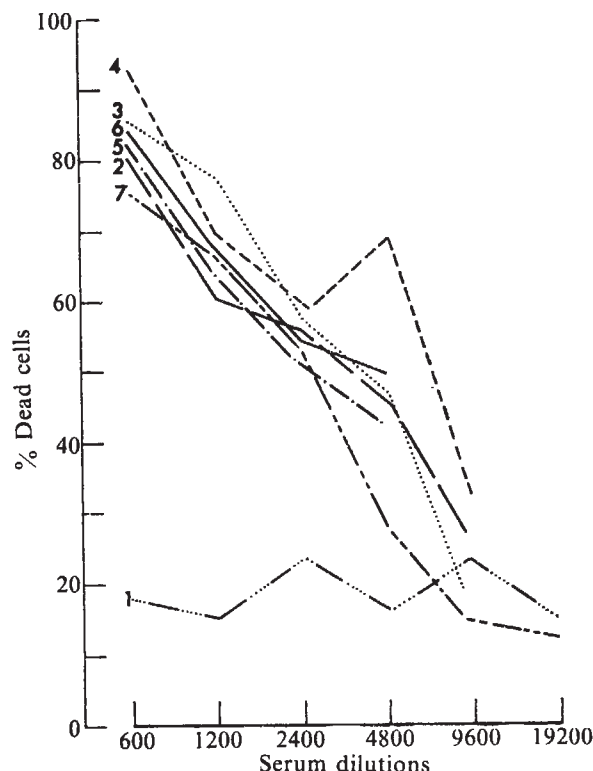


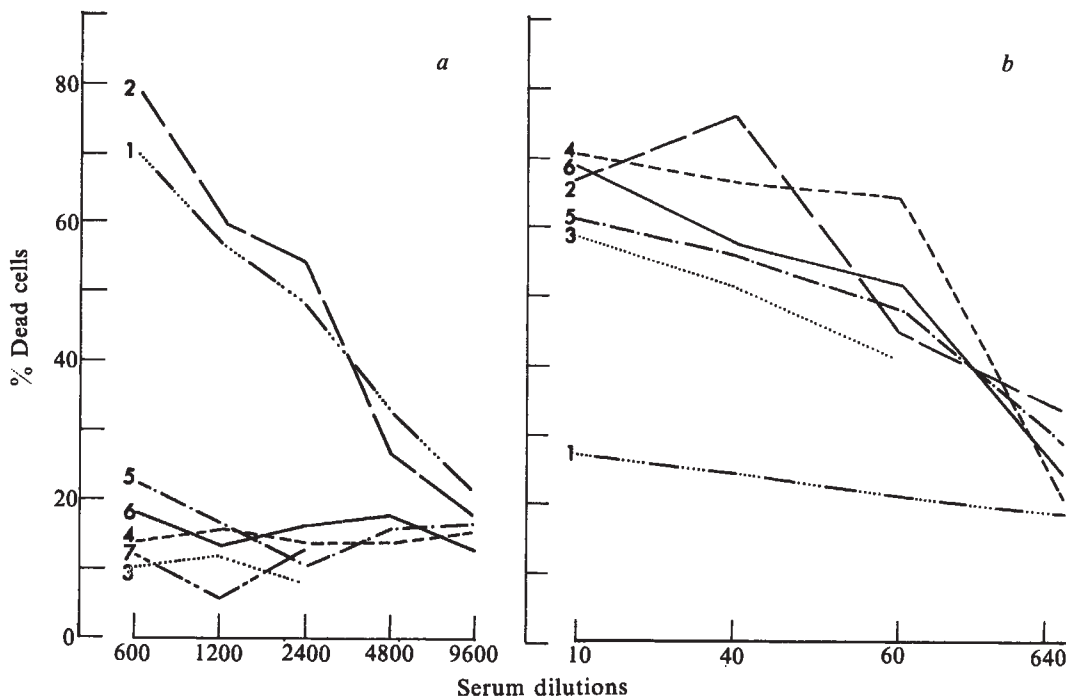
Fig. 1 Complement-mediated cytotoxic activity of anti-F9 serum on F9 cells before absorption (2), after double absorption with human EC cells, Tera II (1), Tera I (3), human melanoma, MalME (4), human intestinal tumour HT 29 (5), human lung tumour SK MEM-1 (6) and murine yolk sac carcinoma PYS (7). F9 cells originated from Steven's OTT6050 teratocarcinoma; human cells were isolated and supplied by Føgh⁴. All cells were cultivated at 37 °C in 12% CO₂ in air as a monolayer on plastic Petri dishes in Dulbecco's modified Eagle's medium containing 15% foetal calf serum and 1% penicillin/streptomycin. F9 but not human cells were grown on a 0.1% gelatin layer. The preparation of antisera, complement and cells as well as the details of the cytotoxicity test are identical to the technique previously described¹ except that double absorptions performed at 4 °C for 45 min each utilising a 1:2 ratio of cell volume to serum volume were required for Tera II to remove anti-F9 activity. The absorptions were performed at a serum concentration of 2 doubling dilutions below the titre. Lines 1–3 represent the averages of three to nine independent experiments. Lines 4–7 represent single experiments. All complement and cell controls were between 10 and 22% dead.

Both EC and early embryo cells contain large quantities of alkaline phosphatase which decline with the onset of differentiation⁵. Although the function of this enzyme is unknown, it is a useful marker for cellular differentiation. We tested Tera I and Tera II for the presence of alkaline phosphatase by the method of Burstone⁶. Our findings that Tera II cells contained high levels of alkaline phosphatase plus F9 antigen while most Tera I cells lacked F9 antigen and had low alkaline phosphatase could be explained if most Tera I cells are more differentiated than Tera II.

Because mouse EC or embryonic cells that express F9 antigen lack H-2 (ref. 7), and H-2 becomes detectable with the onset of differentiation as F9 disappears⁸, we tested indirectly for the presence of HLA antigen on Tera II cells. Because we did not know the HLA type of the patients from whom these cells derive, we tested for the presence of β_2 microglobulin, with which the HLA antigens are invariably associated. Complement-mediated cytotoxicity tests and absorption showed that Tera II did not express β_2 microglobulin, while Tera I and other human cell lines tested did (Figs 2 and 4). This suggests not only that Tera II lacks HLA but also that it lacks the human counterpart of other mouse antigens such as TL and QA-2, which are also associated with β_2 microglobulin in the membrane.

Two observations made in patients with testicular cancer suggest that human teratocarcinoma does not stimulate the host

Fig. 2 *a*, Direct complement-mediated cytotoxic activity of murine anti-F9 serum on Tera II (1), F9 (2), SKMEM-1 (3), Ma1ME (4), Tera I (5), human fibroblasts (6) and HT29 (7). Lines 1-4 represent the averages of two to nine independent experiments. Lines 5-7 represent single experiments. Complement controls averaged 23.8% dead and were all below 30%. Cell controls averaged 16.4% dead and were all below 25%. *b*, Direct complement-mediated cytotoxic activity of rabbit anti-human β_2 microglobulin (DAKO-immunoglobulins code no. 10-U72, Dakopatts, Denmark) on Tera II (1), fibroblasts (2), Tera I (3), HT-29 (4), SKMEM-1 (5) and Ma1ME (6). Lines 1, 2 and 6 represent averages of two independent experiments. Lines 3-5 represent single experiments. Complement, serum and cell controls ranged from 9 to 28% dead and averaged 18.4%.



cellular immune response. First, unlike patients with virtually all other forms of cancer, those with testicular tumours in advanced stages demonstrate a grossly intact cellular immune system when measured by their ability to respond by delayed cutaneous

hypersensitivity to intradermal antigens such as dinitrochlorobenzene⁹. Second, unlike the case of patients with various cancers tested, the immunomorphological features of the regional lymph nodes in testicular cancer have not been correlated successfully with prognosis¹⁰. The lack of HLA on teratocarcinoma may explain these anomalies. For example, the absence of H-2 on F9 cells apparently prevents them from serving as target cells either for allogeneic cytotoxic T cells immunised against spleen cells syngeneic to F9, or for syngeneic T cells sensitised to trinitrophenyl- or virus-modified spleen cells^{11,12}. It has been suggested that the phenotypic expression of *H-2* genes by the target cell is necessary for cell-mediated immunity^{12,13}. Thus T cells in regional lymph nodes of human patients may be unable to see EC cells because of their lack of HLA expression. This hypothesis, if correct, could have direct impact on plans for immunotherapy of this disease.

Because embryonic antigens (F9) stimulate a humoral or 'autoantibody' response in syngeneic systems, and human teratocarcinomas express F9 antigen, our current efforts are directed toward the detection of anti-F9 autoantibody in the sera of

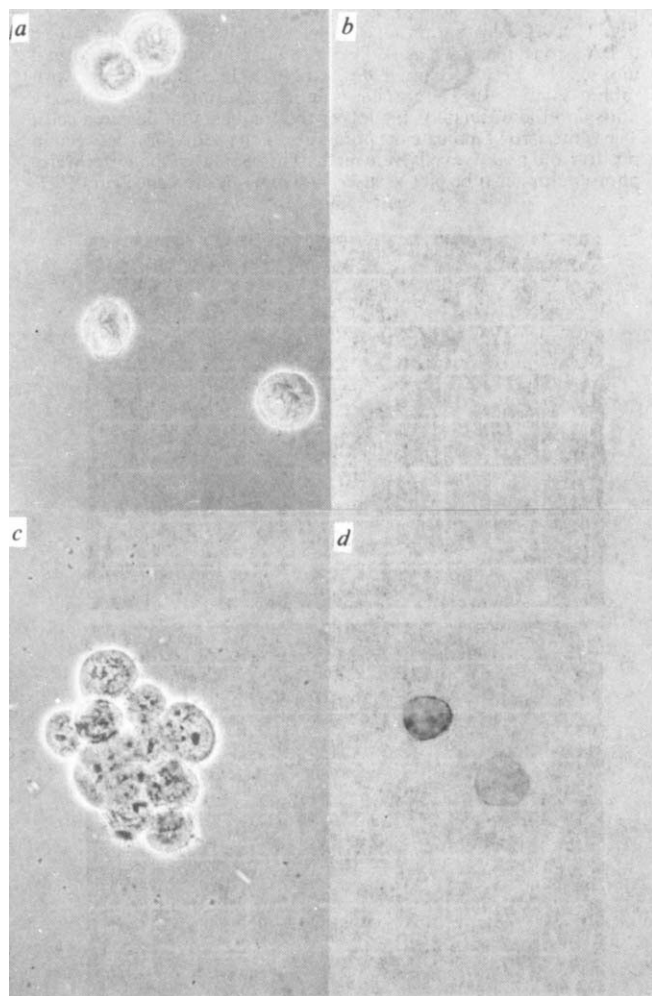


Fig. 3 Immunoperoxidase study of anti-F9 serum on human teratoma cell lines. $1-2 \times 10^6$ cells were incubated for 60 min at $+4^\circ\text{C}$ in 0.1 ml anti-F9 serum diluted 1:50 in Hanks medium supplemented with 5% heat-inactivated γ -globulin-free foetal calf serum (Gibco). Cells were washed three times in the same medium and resuspended in 0.1 ml of a $65 \mu\text{g ml}^{-1}$ solution of peroxidase-labelled sheep Fab anti-mouse immunoglobulins (Institut Pasteur) for another 1 h at $+4^\circ\text{C}$. After three washings in medium, cells were fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS), further washed in PBS and stained for peroxidase activity in diaminobenzidine (50 mg per 100 ml in Tris-HCl, pH 7.5) and 0.01% H_2O_2 for 3 min at room temperature. *a*, Tera II cells incubated in anti-F9 antiserum, phase contrast micrograph. *b*, Tera II cells incubated in anti-F9 antiserum. A discrete labelling of the cell membrane was detectable in 80% of the cells. No labelling was present when anti-F9 serum had been previously absorbed on F9 cells. Absorption of anti-F9 serum on PYS cells did not alter the labelling observed with unabsorbed serum ($\times 350$). *c*, Tera I cells incubated in anti-F9 serum. Phase contrast micrograph. *d*, Tera I cells incubated in anti-F9 serum. Most of the cells were unlabelled, but about 15% showed a membrane labelling sometimes stronger than that on Tera II cells. This labelling was no longer detectable with anti-F9 serum absorbed on F9 cells ($\times 350$). When Ma1ME cells were incubated with the same anti-F9 serum, some cell membrane labelling could be detected. This, however, was not removed by absorption of anti-F9 serum on F9 cells.

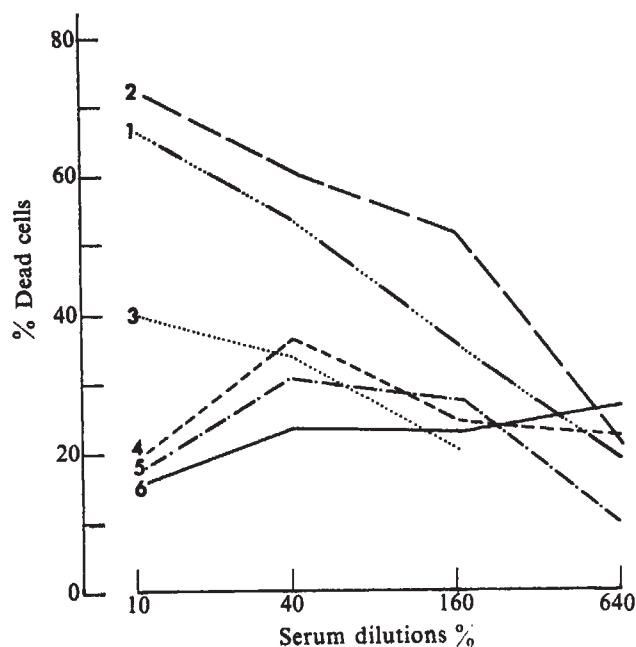


Fig. 4 Complement-mediated cytotoxic activity of rabbit anti-human β_2 microglobulin on human fibroblasts before absorption and (2) after single absorption with Tera II (1), Tera I (3), HT 29 (4), SKMEM-1 (5) and Ma IME (6). Lines 1 and 3 represent the averages of two independent experiments. Lines 3–6 represent single experiments. All complement, serum and cell controls varied between 8 and 28% dead.

patients with testicular cancer. If present, this activity could serve as a highly specific biological marker of tumour activity.

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STUART HOLDEN
OLIVIER BERNARD
KAREN ARTZT
WILLET F. WHITMORE, JR
DOROTHEA BENNETT

Departments of Developmental Genetics and Urology,
Memorial Sloan-Kettering Cancer Center,
New York, New York 10021

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Murine pluripotential stem cells lack Ia antigen

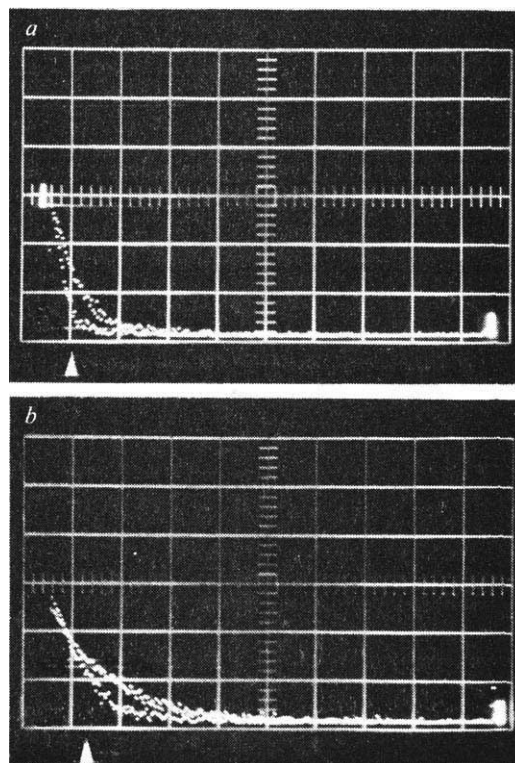
THE Ia (I region associated) antigens of the mouse were first described as differentiation antigens of B lymphocytes^{1,2}. Subsequently, similar antigens, determined by the same or closely linked genes, have been found on macrophages³, on some T cells⁴ and on stable products released by T cells⁵. Immunoprecipitated Ia antigen is a dimeric molecule, consisting of two dissimilar polypeptides⁶. The alloantigenic polymorphism of the structure is attributed to variation in the larger (33,000 molecular weight)

subunit. Evidence has accumulated suggesting that Ia antigens are involved in immunological recognition and/or cell interactions^{8,9}.

Structures with similar biochemical properties have been identified on human cells and specific hetero- and allo-antisera against these Ia-like antigens are available^{10–14}. The distribution of the human 'Ia-like' material on normal cells closely parallels that found in mice. Cells in chronic lymphocytic leukaemia and B type lymphomas have been found to be positive^{11–16}. Unexpected and intriguing results were obtained, however, when the distribution of these antigens on leukaemic and foetal cells was studied. Most leukaemic lymphoblasts in patients with the common, non-T, non-B type of acute lymphoid leukaemia (ALL), myeloblasts in patients with acute myelocytic leukaemia (AML) and blast cells found in patients with chronic myeloid leukaemia in blast crisis (CML-BC) expressed a Ia-like antigen which was completely cross-reactive with B cell Ia (refs 14–16). More mature myeloid cells (myelocytes and granulocytes) on the other hand were Ia negative^{16,17}. Ia positive, membrane immunoglobulin negative, lymphoblasts, myeloblasts and in some cases promyelocytes, were also found in non-leukaemic adult and foetal bone marrow cells^{16,17}. On this basis it was suggested that 'Ia-like' antigens on blast cells in acute leukaemia may reflect their origin from Ia positive haematopoietic stem cells^{16,17}.

A direct assay for cells which serve as a precursor to both lymphocytes and granulocytes is not available in man. These pluripotential stem cells can, however, be quantified in the mouse. Transfer of limited numbers of haematopoietic cells into irradiated mice results in the formation of discrete nodules in the spleen¹⁸. These spleen colonies are the product of the clonal proliferation of pluripotential stem cells (CFU_s). We have examined the effects of antisera to Ia antigens on the cells which give rise to spleen colonies.

Fig. 1 FACS histogram analysis of Ia positive cells from mouse (CBA) bone marrow. *a*, A.TH α A.TL compared with normal mouse (A.TH) serum; *b*, rabbit α Ia compared with rabbit α mouse Ig. The abscissa is fluorescence intensity in arbitrary units and the ordinate is the log of the frequency of detected cells. The white arrows indicate the points at which the division between Ia positive and negative cells were made. The settings of the sorter were: photomultiplier tube 600 V, laser, 400 mW, fluorescence gain 16:1, scatter gain 2/0.5.



The results in treating mouse bone marrow cells with polyvalent alloantiserum against Ia (A.TH $\times$ A.TL) and complement are shown in the first experiment in Table 1. The anti-Ia serum did not reduce the number of colonies found. Line 3 of the Table shows that the CFU of CBA mice can be killed by antibody (rabbit anti-mouse brain) and complement in the conditions used. Since the failure of alloantiserum to kill is not proof of the absence of antigen, we used the fluorescence-activated cell sorter (FACS I) to separate Ia⁺ and Ia⁻ cells from mouse bone marrow and tested these cells for their ability to form spleen colonies. To prevent the complement mediated destruction of antibody-coated cells after their injection into the irradiated recipients the cells were incubated at 37 °C for 1 h in tissue culture medium RPMI1640 supplemented with 2% heat inactivated foetal bovine serum. This treatment effectively removes the antibody both as judged by fluorescence microscopy and by the recovery of CFU_(s) from cells treated with rabbit anti-mouse brain (Fab₂') and fluoresceinated goat anti-rabbit Ig (lines 10 and 11, experiment 2, Table 1). 14.1% of the total are Ia positive on the basis of the sorter analysis (compared to 6.0% when stained with normal mouse serum) as shown in Fig. 1a. Both the positive cells and the negative cells were collected and injected into irradiated recipients. The results are shown in experiment 2, Table 1. Almost all of the CFU_(s) of C₃H bone marrow was recovered in the Ia⁻ fraction. The apparent activity of the Ia⁺ fraction (line 8) can be accounted for by

Table 1 Effect of anti-Ia alloantiserum on CFU_(s) obtained from mouse bone marrow

Expt 1: negative selection with anti-Ia antiserum and complement

Antiserum	Volume per 10 ⁶ cells (μl)	Spleen colonies per 10 ⁵ cells injected
1. Normal A.TH serum	50	17.4 ± 2.6 (14)
2. A.TH $\times$ A.TL	50	16.3 ± 3.5 (12)
3. Normal rabbit serum	20	15.8 ± 3.1 (5)
4. Rabbit anti-mouse brain	20	2.2 ± 1.7 (5)
5. No cells transferred		0.3 ± 0.1 (18)

Expt 2: Positive selection using FACS to obtain Ia⁺ cells

Cells transferred	Number	Spleen colonies
6. Unfractionated CBA bone marrow	100,000	18.2 ± 2.1 (9)
7. Ia negative fraction	100,000	15.6 ± 3.9 (8)
8. Ia positive fraction	50,000	5.8 ± 1.6 (4)
	15,000	1.7 ± 1.5 (5)
9. Unfractionated + NRS	100,000	14.0 ± 2.8 (5)
10. Unfractionated + R anti-MB + C	100,000	4.2 ± 2.6 (5)
11. Unfractionated + R anti-MB + goat anti-R Ig incubated at 37 °C for 1 h	100,000	14.7 ± 4.0 (5)
12. No cells transferred	0	0.4 ± 0.2 (5)

Recipient mice (CBA in expt 1, C₃H in expt 2) were irradiated with 750 R from a ⁶⁰Co source and injected intravenously with bone marrow cells suspended in tissue culture medium. The animals were killed 9 d later and the spleens removed and fixed in Bouin's solution. The spleen colonies visible on the surface were counted under a dissecting microscope at 25 $\times$ magnification. In Expt 1, 10⁶ bone marrow cells were incubated at 4 °C with 20–50 μl of antiserum in a total volume of 250 μl. After 1 h they were washed twice and then incubated for 30 min at 37 °C with guinea pig serum diluted 1:3. The cells were then washed twice, resuspended in media and 10⁵ cells injected into each mouse. A.TH $\times$ A.TL, prepared by Dr I. McKenzie, was obtained by Dr Marc Feldmann and had a titre of ~ 1:50 against CBA spleen cells. R $\times$ MB was prepared as previously described and had a titre of 1:200 (ref. 19). It had been absorbed with mouse red blood cells, liver and cortical thymocytes from CBA mice. In expt 2, 10⁷ cells were incubated with 100 μl of antiserum in a total volume of 250 μl at 4 °C for 30 min, washed twice and then incubated for 30 min with 50 μl of a 1 μg ml⁻¹ solution of the fluoresceinated (Fab₂') fraction prepared by pepsin treatment of the total immunoglobulins of rabbit anti-mouse Ig serum. They were then washed twice and passed through the FACS. All of the FACS-passed and control cells were adjusted to 10⁶ cells ml⁻¹ and incubated at 37 °C for 1 h before being injected into the irradiated mice.

*Values are means $\pm$ s.d.; number of tests performed is given in parentheses.

Table 2 Effect of heterologous antiserum to mouse Ia on the CFU_(s) obtained from mouse bone marrow

Expt 1: negative selection with anti-Ia and complement

Antiserum	Volume per 10 ⁶ cells (μl)	Spleen colonies per 10 ⁵ cells injected
1. Normal rabbit serum (pre bleed)	50	14.8 ± 1.6 (5)
2. 'Anti-Ia'	50	13.5 ± 2.8 (5)
3. 'Anti-Ia' absorbed with mouse Ig on BioGel bleeds	150	14.6 ± 1.9 (4)
4. Anti-mouse Ig	50	11.9 ± 3.5 (5)
5. Complement only	—	12.2 ± 2.6 (6)
6. No cells	—	0.2 ± 0.1 (4)

Expt 2: Positive selection

Cells transferred	Number	Spleen colonies
7. Unfractionated CBA bone marrow	100,000	12.7 ± 3.1 (5)
8. 'Anti-Ia' negative cell	100,000	17.3 ± 4.2 (4)
9. 'Anti-Ia' positive cell	100,000	5.1 ± 2.7 (4)
10. No cells	—	0

Condition as for Table 1. The 'anti-Ia antiserum' after exhaustive absorption with insolubilised MIg stained ~ 35% mouse spleen cells and ~ 5% of mouse bone marrow cells at a dilution of 1:20 when developed with a fluoresceinated goat anti-rabbit Ig serum (Fab₂').

contamination of the Ia⁺ fraction with Ia⁻ cells. The sorter was adjusted so that positive cells were excluded from the negative fraction but many cells which lacked Ia antigen were included in the Ia positive fraction.

Most of the studies of human 'Ia-like' antigens on human cells have used heteroantisera against Ia. Since these sera recognise only the non-polymorphic subunit⁷ it is possible that only this portion of the molecule is present on the stem cells. They would thus escape detection by the alloantiserum used in the experiments reported in Table 1. To obviate this we have prepared a heterologous antiserum to mouse Ia antigens by injecting immunoprecipitates of Ia-alloanti-Ia complexes (precipitated with rabbit anti-mouse Ig) into rabbits. After four injections at 2-week intervals a serum was obtained which seems to contain anti-Ia antibodies. This serum, after exhaustive adsorption with insolubilised mouse Ig reacts with the majority of the Ig⁺ cells of the spleen and bone marrow and also stains some Ig⁻ cells from both organs. A detailed report of its properties will be presented elsewhere. Its effect on the mouse bone marrow cells which produce spleen colonies is shown in Table 2. In the first experiment the serum was added to CBA bone marrow cells with an effective source of complement and in the second FACS-separated C₃H cells were injected: 19.6% of the cells are antigen positive compared with a background of 12.8% with normal rabbit serum (Fig. 1b). The results indicate that the cells identified by this heterologous antiserum to mouse Ia antigen are not CFU_(s). The serum does not kill CFU_(s) and essentially all of the CFU are recovered in the 'Ia' negative fraction.

If these results can be generalised to man, it would seem that the cells recognised by the anti-'Ia' sera in human foetuses and in AML are not related to the most immature haematopoietic elements.

Ia antigens may only be expressed on progeny of these cells: cells which are already committed to either the lymphoid or myeloid cell lineages. Preliminary observations in man suggest that both Ia⁺ and Ia⁻ populations do contain myeloid precursors which form colonies when grown *in vitro* in semi-solid media (CFU_(c))²⁰ (G.J., X. Francis and M.F.G., unpublished). The basis for this heterogeneity has not been established but it seems possible that it is a consequence of the presence of cells of different degrees of maturity in the CFU_(c) population.

Alternatively, it has been suggested that the expression of human Ia-like antigens is cell cycle dependent. CFU_(s) in the mouse are not a mitotically active population. It is thus possible that

multipotential stem cells when driven into cell division might express Ia antigens but the resting cell normally present in the bone marrow seems to lack this antigen.

R. S. BASCH*
G. JANOSSY
M. F. GREAVES

ICRF Tumour Immunology Unit,
Department of Zoology,
University College London
and
Membrane Immunology Laboratory,
Imperial Cancer Research Fund

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*Present address: Department of Pathology, New York University Medical Center, 550 First Avenue, New York, New York 10016.

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Absence of suppressor cells from rats bearing passively enhanced kidney allografts

LYMPHOCYTES which inhibit the immunological responses of other lymphocytes are known as suppressor cells, and such cells have been shown to mediate both specific and non-specific unresponsiveness in many experimental situations¹⁻³. In the context of allotransplantation, Kilshaw *et al.*^{4,5} have found that the highly strain-specific unresponsiveness to skin allografts induced in adult mice by pretreatment with donor strain tissue extract and *Bordetella pertussis* vaccine, followed by a short post-operative course of antilymphocyte serum (ALS), is at least in part mediated by thymus-dependent lymphocytes. Thus, transfer of $25-100 \times 10^6$ spleen cells (optimally the lower dose) from mice in the stable phase of unresponsiveness into ALS-treated syngeneic recipients established a long-lasting unresponsiveness to donor strain skin grafts in up to 50% of recipients. Because the nature of the mechanisms of immunological enhancement of kidney allografts in rats remain unclear⁶⁻⁸ we have carried out cell transfer experiments similar to those of Kilshaw *et al.* in order to discover whether suppressor cells also play a part in mediating the stable phase of the enhanced state. Fabre and Morris⁹ have previously failed to transfer unresponsiveness with spleen cells from rats with enhanced kidneys, but it is possible that transfer of cells to normal, syngeneic rats—as in their experiments—is an insufficiently sensitive method for detecting suppressor cells. Our results show that it is unlikely that suppressor cells or auto-anti-idiotypic antibodies are responsible for the steady state of enhancement. A more likely explanation involves the induction of unresponsiveness by the continuous exposure of the recipient to histocompatibility antigens in the absence of Ia-like antigens—the latter determinants being obligatory requirements for the activation of T cells 'help-

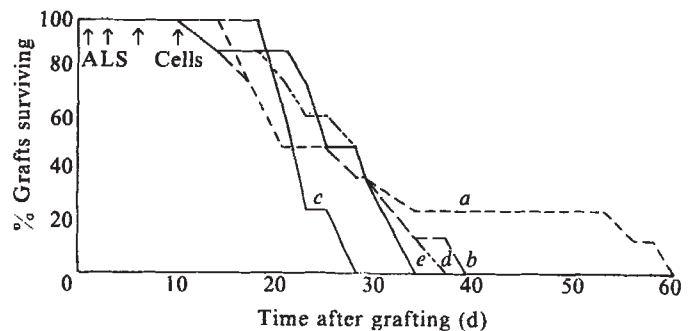


Fig. 1 Survival of AUG skin grafts in ALS-treated AS males following transfer of spleen and lymph node cells from normal AS female rats or female AS donors that had received passively enhanced F1 kidneys 5-9 months before (experiment 1). Cells were injected i.p. on day 10, ALS s.c. on days 1, 3 and 6 (arrows). ALS dose, 4 ml per rat per injection: 8 rats per group. *a*, Received 175×10^6 cells from donors with enhanced kidneys. *b*, Received 25×10^6 cells from donors with enhanced kidneys. *c*, Received 175×10^6 cells from normal donors matched for age and sex. *d*, Received 25×10^6 cells from normal donors matched for age and sex. *e*, ALS controls.

ing' both potentially reactive B cells and T cells capable of differentiating into killer cells.

Passive enhancement of (AS $\times$ AUG)F₁ kidneys transplanted orthotopically to AS strain recipients was induced by injecting the recipients with 0.15-1.0 ml of AS-anti-AUG alloantiserum. As described previously^{6,7}, this treatment leads to the production of a stable unresponsiveness, the majority of recipients continuing to live indefinitely with normal or slightly raised blood urea levels. The cell donors used in the three transfer experiments described here had been sustained by their grafts for 5-14 months, with a mean of 8 months.

The design of the transfer experiments was essentially the same as that used to demonstrate the presence of suppressor cells in the long-term unresponsive mice described by Kilshaw *et al.*^{4,5}. Adult AS rats were grafted with AUG strain skin allografts on day 0; rabbit anti-rat lymphocyte serum (ALS), raised by two intravenous injections of approximately 10^9 AS lymph node cells, was administered subcutaneously to these animals either on days 2, 4 and 6 or on days 1, 3 and 6 after transplantation (for details see figure legends). On day 9 or 10, lymphoid cell suspensions were prepared from the AS rats bearing enhanced kidney allografts and injected in varying numbers into the prepared recipients (intraperitoneally in experiment 1 and intravenously in experiments 2 and 3).

Spleen and lymph node suspensions for transfer were prepared by shredding the tissues with fine forceps in RPMI 1640 medium to which 5% foetal calf serum had been added. The coarse suspension was then sieved and clumps were removed by rapid passage through a cotton wool filter or by allowing the clumps to settle. The resulting single cell suspensions were centrifuged for 10 min at 1,200 r.p.m., resuspended, counted and assessed for viability.

The results were clear-cut. In experiment 1, neither high nor low doses of mixed spleen and lymph node cells transferred unresponsiveness, although 2/8 of the grafts in the high dose group exceeded the median survival time of the control grafts by 20-25 d (Fig. 1). High doses of normal cells somewhat curtailed graft survival, as might be expected. In the second experiment (Fig. 2) only 1/11 grafts in the group receiving spleen cells showed prolonged but limited survival beyond that of the controls; in the third (Fig. 3) there was no difference whatever between experimental and control groups. According to the Mann-Whitney test there were no statistically significant differences between the experimental and control group of experiments 1 and

2 ($P = >0.05$). It must be concluded that splenic suppressor cells do not have a significant role in mediating the stable phase of the enhanced state, unless we have been singularly unfortunate in our choice of the number of cells transferred. The lower dose (Fig. 1) corresponds to that giving optimal results in mice⁵, and the higher dose was intended to take into account the body weight differences between mice and rats.

These experiments also provide evidence against the participation of auto-anti-idiotypic immunity in the 'steady state' of enhanced kidney allografts. Binz *et al.*¹⁰ have shown that B- and T-cell recognition structures have idiotypes in common, and that anti-idiotypic antibodies will combine with subpopulations of T and B cells which react with the same transplantation antigens¹¹. Because auto-anti-idiotypic antibody production can be provoked in Lewis rats either directly by immunisation with purified natural antibody¹² or with specifically reactive autologous T cells¹³, or indirectly by allogeneic tissue¹⁴, kidney allograft survival could be due to the development of auto-anti-idiotypic immunity¹⁵. Whilst auto-anti-idiotypic immunity can undoubtedly be induced in both mice and rats, and can cause a specific unresponsiveness¹⁶, it has yet to be shown that this is the major mechanism which protects kidney allografts during the steady state of enhancement. If auto-anti-idiotypic immunity were involved it should be possible to transfer the unresponsiveness adoptively with lymphoid cells, but our experiments show that this does not happen. Other evidence against the auto-anti-idiotypic hypothesis is provided by the finding that kidney allograft protection cannot be achieved convincingly by actively immunising AS rats with purified AS-anti-AUG antibody derived from enhancing sera⁷, despite the fact that in this strain combination enhancement can be brought about with relatively small doses of antiserum; and by the observation that passive immunisation of Lewis rats with Lewis anti-(Lewis-anti-BN) antibody only occasionally prolongs the survival of (Lewis × BN)F₁ kidney allografts¹⁴.

If neither suppressor cells nor anti-idiotypic antibodies are primarily involved in mediating the stable phase of the enhanced state, what other mechanism is likely to be operative? Cantor and Boyse¹⁷ have shown that T lymphocytes can be divided into at least two populations, one responding to Ia-like antigens and the other maturing into killer T lymphocytes with specificity for the classical histocompatibility (H) antigens. The Ia-responsive population of T cells provides help both for the B cell lineage, which synthesises IgG antibody against the thymus-dependent MHC antigens^{18,19}, and the T-cell clones, which differentiate into

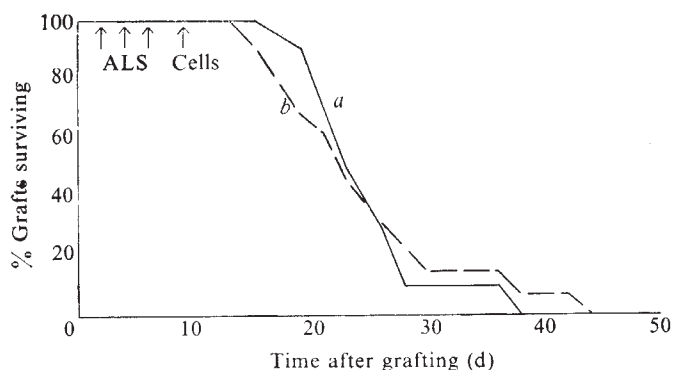


Fig. 3 Survival of AUG skin grafts in ALS-treated AS males following transfer of spleen cells from male AS donors that had received their passively enhanced F1 kidneys 9–14 months before (experiment 3). Cells injected i.v. on day 9, ALS s.c. on days 2, 4 and 6 (arrows). ALS dose, 1.0 ml per 40 g body weight per injection. a, Received 200×10^6 cells from donors with enhanced kidneys (11 rats). b, ALS controls (13 rats).

killer T lymphocytes with specificity for H-type antigens^{20,21}. Because kidneys contain little or no Ia-type antigen²², and long-surviving allografts must be devoid of donor-type passenger leukocytes, during the steady state of enhancement the recipient is exposed to a continuous presence of H-type antigen in the absence of stimulation by Ia. We postulate that this exposure not only fails to provoke immunity but also induces cumulatively a specific non-reactivity of the T and B precursor cells which, if provided with 'help', would normally have differentiated into T killers and antibody producers, respectively, with specificity for H-type antigens.

In keeping with this hypothesis is the finding that pretreatment of rats with allogeneic platelets leads to suppression rather than activation of humoral immunity²³, for platelets possess H antigens but lack Ia. Consistent depression of killer T lymphocyte generation after platelet injection was not observed, but only a limited number of dose schedules have so far been examined. Because prolonged kidney allograft survival has been observed after pretreatment of recipients with platelets²⁴, and Wagner and Nossal²¹ have shown that cellular immunity generated *in vitro* can be specifically suppressed by allogeneic membranes provided that high doses are added to the cultures, the question of dosage effects *in vivo* clearly needs further examination.

It has been suggested that induction of enhancement is due to the opsonisation of antigen-reactive cells by antigen-antibody complexes and their subsequent removal through phagocytosis²⁵. The maintenance of the steady state by this mechanism would require the continuous production of anti-donor antibody. Because the amount of antibody required for maintenance (as opposed to induction) may be quite small our transfer experiments do not necessarily exclude this possibility.

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J. R. BATCHELOR

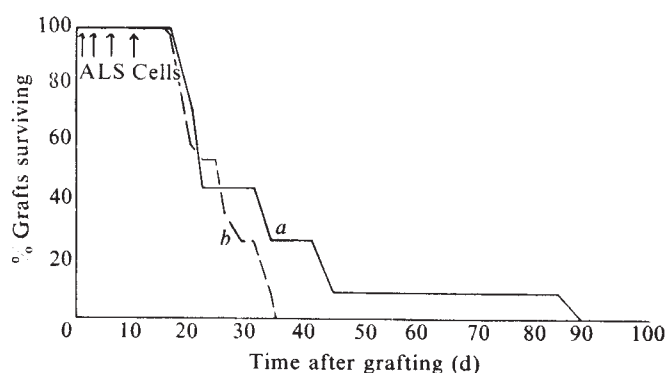
McIndoe Research Unit,
Queen Victoria Hospital,
East Grinstead,
Sussex, UK

L. BRENT
P. J. KILSHAW*

Department of Immunology,
St Mary's Hospital Medical School,
London W2, UK

Received 15 August; accepted 11 October 1977.

Fig. 2 Survival of AUG skin grafts in ALS-treated AS females following transfer of spleen cells from female AS donors that had received their passively enhanced F1 kidneys 5–8 months before (experiment 2). Cells injected i.v. on day 10, ALS s.c. on days 1, 3 and 6 (arrows). ALS dose, 1.0 ml per 50 g body weight per injection: 11 rats per group. a, Received 200×10^6 cells from donors with enhanced kidneys. b, ALS controls.



* Present address: Nutrition Department, National Institute for Research in Dairying, Shinfield, Reading, Berkshire, UK.

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HLA restriction of cell-mediated lysis of influenza virus-infected human cells

MURINE T lymphocytes that mediate the lysis of virus-infected cells show specificity both for the viral cell surface antigens and for the H-2K or D antigens of the major histocompatibility complex¹⁻⁸. The cytotoxic T lymphocytes and the target cell must share H-2K or D products. The experiments reported here demonstrate that there is a similar requirement for partial HLA identity between human cytotoxic lymphocytes and influenza virus-infected target cells.

The viruses used in this study were influenza type A/X31 (A₂/Hong Kong/68×A₀/PR8 (H3N2) and type B/Hong Kong (B/Hong Kong/1/73). H-2 compatibility has been shown to be required for T-lymphocyte-mediated lysis of influenza virus-infected target cells in mice³⁻⁸. Although there was no cross reaction between type A and B influenza viruses, type A viruses with serologically distinct surface proteins were found to cross react both at the level of secondary induction of cytotoxic cells and in the recognition of infected target cells⁷⁻⁹. Consequently, we anticipated that deliberate immunisation of the human volunteers studied would not be necessary, because nearly

all adults have been infected with some influenza viruses of the A group. Similarly, most would have been exposed to viruses of the B type. The individuals studied were therefore asked only to give venous blood samples.

Influenza viruses grown on chick allantoic membranes were used to infect peripheral blood lymphocytes, prepared from heparinised blood by centrifugation on Ficoll-Hypaque. These cells were exposed to virus and after a further 4-h incubation they could be lysed by rabbit antibody to type A influenza virus and complement and therefore expressed viral antigens on their surface. Such infected cells served as stimulator cells for *in vitro* sensitisation or target cells for cell-mediated lysis (CML) (ref. 10). A similarly infected lymphoblastoid cell line (PGF) was also a good target.

Cytotoxic cells could be generated by sensitising peripheral WBC with influenza virus-infected autologous cells, *in vitro*, for 5-8 d at 37 °C. Cell-mediated lysis was then assayed for 5 h on a panel of freshly prepared peripheral blood lymphocytes which were virus-infected and ⁵¹Cr-labelled. These conditions were found to be optimal in preliminary experiments with killer target cell ratios of 40:1 or 50:1. After sensitisation with influenza virus type A/X31 or B/Hong Kong, peripheral blood lymphocytes from J.D. and F.W. killed infected autologous cells, and cytotoxicity was specific for the immunising virus type A or B (Table 1). C.W. cells could be sensitised to A/X31 but not to B/Hong Kong which may indicate that C.W. had not been previously exposed to the B/Hong Kong virus. Otherwise it was apparent that previous exposure to virus *in vivo* had resulted in immunological priming or, less likely, that the cytotoxicity generated *in vitro* represented a primary immune response. Freshly prepared peripheral blood lymphocytes showed no cytotoxic activity towards infected cells and therefore a period of sensitisation *in vitro* was essential. The finding that sensitised J.D. and F.W. cells showed specificity for the virus used for sensitisation only, also confirmed this observation.

The infected target cells used in the cytotoxic assay had been incubated long enough to express viral antigens at the cell surface. Zweerink and Askonas (unpublished data) have shown in the mouse, that this was required before influenza-infected cells could be lysed by cytotoxic T

Table 1 Human cytotoxic lymphocytes show specificity for influenza virus type A or B

Cell donor	Sensitised to influenza type	% ⁵¹ Cr release* from target cells infected with		
		A/X31	B/Hong Kong	Uninfected
J.D.	A/X31	36.0	20.2	18.2
	B/Hong Kong	27.3	37.0	18.4
	Medium control	21.0	19.5	15.5
F.W.	A/X31	35.5	21.3	15.5
	B/Hong Kong	20.5	35.4	15.5
	Medium control	16.0	17.7	15.1
C.W.	A/X31	52.8	13.1	10.3
	B/Hong Kong	29.4	19.4	11.3
	Medium control	16.7	9.9	10.5

*% ⁵¹Cr release = (counts released by target cells/counts released by 2.5% Triton X-100) × 100. ⁵¹Cr-release assay was carried out in triplicate in microtitre II plates¹⁰ using 10⁴ virus-infected ⁵¹Cr-labelled peripheral blood lymphocytes and 5 × 10⁵ immune cells. Incubation was for 5 h.

Target cells were prepared on the day of the CML assay by infecting peripheral blood lymphocytes with influenza virus type A/X31 or B/Hong Kong (see below). Target cells were radiolabelled by the addition of 100 μCi ⁵¹Cr during the period of infection (90 min), excess ⁵¹Cr was removed by washing cells three times and incubation was continued for 4 h at 10⁶ cells ml⁻¹ in RPMI 1640 medium-10% FCS. J.D. and F.W. were each tested against autologous target cells, and C.W. against HLA compatible target cells. Human peripheral blood lymphocytes were prepared from heparinised venous blood by centrifugation on Ficoll-Hypaque. The cells at the interface were collected and the red blood cells lysed by a 20-min incubation with Tris-buffered 0.75% ammonium chloride at 37 °C. The cells were then washed three times in RPMI 1640 medium (containing 25 mM Hepes buffer, 200 mM glutamine, penicillin and streptomycin). An aliquot of 3 × 10⁶ lymphocytes was incubated in 0.2 ml with 100 HA units of influenza virus (A/X31 or B/Hong Kong) at 37 °C for 90 min. After a single wash the cells were incubated for 4 h at 37 °C in 3 ml of RPMI 1640 medium containing 10% foetal calf serum (FCS). They were then washed three times and added to the remaining uninfected peripheral blood mononuclear cells at a ratio of 1:20. The cells were incubated at 10⁶ ml⁻¹ in 4-ml volumes in Bijou bottles (Sterilin) in RPMI 1640 10% FCS for 5-8 d at 37 °C in 5% CO₂ air.

Table 2 Human cytotoxic lymphocytes show specificity for HLA

Cytotoxic cell	% Lysis of influenza A/X31-infected target cells*		
	J.D. (3, 7, W2/2, 7, W2)	F.P. (3, 7, W2/1, 8, -)	F.W. (1, 8, -/1, 8, W3)
C.W. (9, 7, W2/9, 7, W1)	43	30	5
F.W. (1, 8, -/1, 8, W3)	6	16	23

C.W. and F.W. peripheral blood lymphocytes were sensitised to their own lymphocytes infected with influenza type A/X31, and were then tested for cytotoxicity against the influenza type A/X31-infected target cells shown. The HLA types are shown for each individual in the sequence A, B, D/A, B, D.

$$*\% \text{ Lysis} = \frac{{}^{51}\text{Cr release} - \text{medium control } {}^{51}\text{Cr release}}{({}^{51}\text{Cr release by Triton X 100}) - (\text{medium control } {}^{51}\text{Cr release})} \times 100$$

lymphocytes. Similarly, sensitised human cells failed to lyse autologous cells exposed to ultraviolet-irradiated virus⁹.

Having established that cytotoxic cells show virus specificity the possibility that their activity is HLA-restricted was explored. HLA A, B and C typing was carried out by the standard Terasaki-NIH microcytotoxicity technique. All the individuals studied had also been typed as normal panel blood donors, for B cell (Ia) alloantigens by sera exchanged in the UK regional workshops¹¹ and Seventh

International workshop. The D locus types thus assigned are those of the UK groups which have been found to correlate with the D specificities defined by mixed lymphocyte typing¹¹. The DWI-3 terminology of the Sixth Workshop¹² is therefore retained.

Effector cells specific for influenza type A/X31 were generated from C.W. (HLA 9,7,W2/9,7,W1) and F.W. (HLA 1,8,W3/1,8,-) and these were tested against virus-infected target cells prepared from C.W., F.W. and F.P. (HLA 3,7,W2/1,8,-) (Table 2). Cytotoxic cells from each individual killed the autologous or partially HLA-matched cells but not the HLA-incompatible cells. Each target cell population tested was lysed by at least one effector, which excluded the possibility that a target might be inadequately infected.

This HLA restriction was explored further using C.W. as the effector cell. C.W. lymphocytes sensitised to A/X31 virus were tested against A/X31 virus-infected peripheral blood lymphocytes from 15 individuals, and one lymphoblastoid cell line (P.G.F.). Figure 1 shows the lysis and its correlation with the degree of HLA compatibility between the target cells and C.W. Infected lymphocytes which shared HLA B7 with C.W. were killed—mean percentage lysis 38.1 ± 1.9 s.e.m. Targets that lacked HLA B7 showed minimal lysis; mean $9.0\% \pm 2.6$ s.e.m. This difference was highly significant: (Students' t 10.8 $P < 0.0001$). When the target cells shared HLA A9 (W24) or the D locus products DW1 or DW2 only, lysis was not observed.

It is therefore apparent that sharing of HLA B7 with the effector cell was sufficient to allow lysis, whereas sharing of the A or D locus products with C.W. was insufficient. Because it was not feasible to test each target-cell preparation with its autologous sensitised cells, with the exception of F.W. (Table 2), it is theoretically possible that only the HLA B7 positive cells were adequately infected. In previous experiments, however, sensitised cells from eight individuals who were HLA B7 negative lysed autologous influenza A/X31 infected target cells, so that the infection of cells is reproducible.

These findings therefore extend to the human the original observations of Zinkernagel and Doherty¹³ that, in the mouse, lymphocyte-mediated lysis of virus-infected cells is restricted by the major histocompatibility complex. This phenomenon is associated with the H-2K and D products which are chemically homologous to HLA A and B antigens¹⁴. It is therefore appropriate that the restriction involves HLA B in man and that HLA D, which is probably equivalent to the H2 I region, was not involved. It is not clear why in the one pair tested, sharing of the A locus antigen A9 (W24), did not allow lysis. Goulmy *et al.*¹⁵ have found, however, that a human (female) cytotoxic cell population sensitised to the H-Y antigen killed targets that shared an HLA A antigen. It seems, therefore, that HLA B and A are functionally equivalent to H-2K and D. Failure to observe lysis when HLA A9 (W24) was shared might therefore be an effect specific to influenza antigen. Alternatively, the A9 (W24) type might include sub-

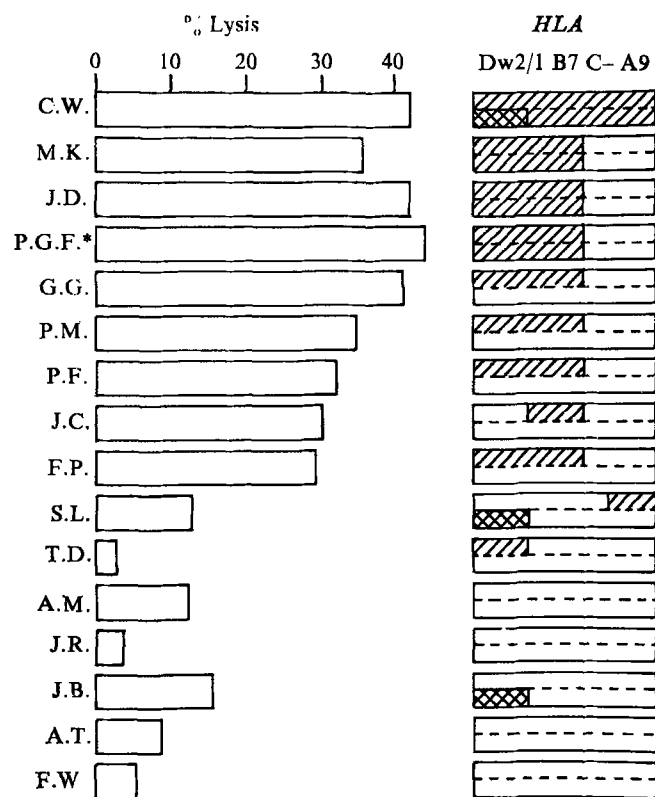


Fig. 1 HLA restriction of cell-mediated lysis of influenza type A/X31-infected cells. C.W. peripheral blood lymphocytes were sensitised to autologous lymphocytes infected with influenza type A/X31, and tested for cytotoxicity against the target cells shown. Results shown as percentage lysis (see legend to Table 2) in the left-hand side of the figure. The degree of HLA compatibility is shown on the right-hand side of the diagram. Sharing of HLA DW2, or B7, C-, A9 is represented by diagonal hatching either as one haplotype (top half only) or two haplotype compatibility (both halves). Sharing of HLA DW1 is shown by cross-hatching. The HLA A, B, D types of the target cells were C.W.: (autologous target). 9(W24), 7, W2/9 (W24), 7, W1; M.K.: 3, 7, W2/3, 7, W2; J.D.: 3, 7, W2/2, 7W2; *P.G.F.: 3, 7, W2/3, 7, W2; G.G.: 2, 3, 7, W14, -, W2; F.P.: 3, 7, W2/1, 8, -; S.L.: 9(W24), W30, 13, W15, W1, -; T.D.: 2, 2, 12, W40, 2, -; A.M.: 2, 21, -/W32, W40, -; J.R.: 2, 2, 12, 27, -, -; J.B.: W30, W32, W15, -, W3, W1; A.T.: 2, 11, W40, W22, -, -; F.W.: 1, 8, W3/1, 8, -.

*P.G.F. was a lymphoblastoid cell line, donated by Professor W. F. Bodmer.

groups that, although serologically related, might not behave as an identical pair in target-cell recognition. This would be analogous to the failure of H-2^b mouse cytotoxic cells to recognise target cells that share the mutant H-2^{ba} K antigen¹⁶. Indeed, if such mutants are common in the human population, apparent HLA homozygotes that are really wild × mutant HLA heterozygotes would lyse a larger selection of target cells than true homozygotes.

The HLA types of the individuals used as cytotoxic cell donors show linkage disequilibrium. The haplotypes A1-B8-DW3 and B7-DW2 occur in Caucasian populations at frequencies that are much greater than would be expected from the gene frequencies of the individual antigens¹². This means that, if the recognition of target cells involves genes closely linked to HLA-B, these are likely to be the same when killer and target cells share an HLA haplotype. This is another possible explanation for the lack of killing when HLA A9 (W24), which is not in linkage disequilibrium with B7, was shared.

This finding that there is HLA restriction of cell-mediated lysis of influenza virus-infected cells is in accord with data in the mouse showing that for influenza⁵⁻⁸ and other viruses¹⁻⁴ there is H2 restriction. It contrasts with reports that human cytotoxic T lymphocytes showed no HLA restriction in the lysis of Epstein-Barr virus¹⁷ or measles virus¹⁸-infected cell lines.

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A. J. McMICHAEL
A. TING

Nuffield Department of Surgery,
University of Oxford,
Radcliffe Infirmary, Oxford, UK

H. J. ZWEERINK*
BRIGITTE A. ASKONAS

National Institute for Medical Research,
Mill Hill, London NW7 1AA, UK

Received 21 July; accepted 25 October 1977.

*Present address: Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina.

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Human cell-mediated cytotoxicity against modified target cells is restricted by HLA

T-CELL-mediated lysis of virus-infected target cells in mice is restricted by the H-2D and H-2K antigens of the major histocompatibility complex (MHC) in the sense that the cytotoxic T cells are only active against virus-infected target cells sharing H-2D and/or H-2K antigens with the animal in which the killer cells have been raised¹. These findings led to a better understanding of the way in which the MHC regulates certain functions of the immune system², and they have been amply confirmed by further studies in inbred strains of mice and guinea pig which have indicated that restriction by antigens of the MHC is a general phenomenon in the T-cell-mediated immune response³ in these species. Evidence for MHC restriction of the immune response in man, however, was lacking until it was shown that a female patient who had rejected a bone marrow graft from her HLA-identical brother, had cytotoxic cells in her blood, and that the activity of these cells was restricted to male target cells which had the HLA-A2 antigen in common with herself⁴. We show here in a more general way, that the cell-mediated cytotoxicity which can be raised in humans against 'altered self' is restricted by the HLA-A, B and C antigens.

It has been shown that peripheral lymphocytes from subjects who have been sensitised by skin painting with DNCB respond with proliferation when cocultured *in vitro* with autologous irradiated and dinitrofluorobenzene (DNFB)-conjugated lymphocytes⁵. We have investigated the generation of cytotoxic cells in such cultures. Individuals were sensitised to DNCB following their informed consent, as a part of a clinical study of the immune response in a group of patients suffering from various allergic disorders, primarily asthma bronchiale. Within this group, subjects with minor disease activity gave permission to use some of their blood samples for a study not directly related to their disorder. A total of 27 subjects were examined. All showed contact sensitivity against DNCB. This hypersensitivity followed either the application of 2,000 µg DNCB dissolved in 50 µl acetone on the volar surface of each forearm, or subcutaneous injection of 25 × 10⁶ DNFB-treated autologous lymphocytes. All subjects were investigated 2-5 weeks after the sensitisation by the method described in the legend to Table 1. Briefly, mononuclear blood cells were mixed with an equal amount of DNFB-treated autologous mononuclear cells and cultured for 8 d *in vitro*. The cytotoxic capacity of these *in vitro* restimulated cells was then investigated on ⁵¹Cr-labelled, DNFB-treated autologous PHA-stimulated lymphocytes, revealing that 14 of the 27 individuals produced cytotoxic cells against autologous DNFB-coupled target cells. Eight were strongly positive and of these, one could not be retested, and two had lost most of the reactivity when retested 2-3 weeks after the first investigation. The results of retesting the remaining five individuals against selected panels of unrelated allogeneic lymphocytes are given in Table 1. The panels were selected according to the HLA types of the sensitised individuals and represented various degrees of HLA disparity. Freshly explanted lymphocytes from the sensitised subjects had no direct cytotoxic activity, and we have not succeeded in generating DNFB-directed cytotoxic cells *in vitro* with lymphocytes from non-immunised subjects.

It is obvious that cytotoxic activity is directed only against DNFB-coupled target cells. Of these, only autologous targets or targets sharing one or more HLA-A,B antigens with the cytotoxic effector cell were lysed, whereas there was no or (in two cases) only weak activity against totally HLA-A,B different target cells. These results show

Table 1 DNFB-directed CML against autologous and allogeneic DNFB-treated and untreated target cells

		(% Specific ^{51}Cr -release)				(% Specific ^{51}Cr -release)	
		DNFB-treated target	Untreated target			DNFB-treated target	Untreated target
Sens. donor KR*: HLA-A2, B7, 40, Cw3				Sens. donor VH*: HLA-A1, 2, B5, 17 Targets:			
Targets:				Autologous		35	0
Autologous		66	2	A1, 2, B5, 17		35	2
A2, B7, 40, Cw3		54	4	A1, 2, B44, w38, Cw4		36	3
A2, B8, 17		61	1	A3, 9, B5, 17, Cw2		7	-1
A1, 3, B7, 40, Cw3		15	1	Aw19, B12		2	-2
A1, 3, B8, 37		5	1	A25, 26, B18, w21, Cw4		2	-1
A25, 29, B44		3	1				
Sens. donor JR*: HLA-A2, B44, 15, Cw3				Sens. donor MC†: HLA-A1, 25, B17, 18			
Targets:				Targets:			
Autologous		50	3	Autologous		24	1
A2, B44, 15, Cw3		63	-1	A1, B17		19	1
A2, B44, 15, Cw1		46	0	A1, 10, B8		6	0
A2, B8, 17		55	0	A2, w24, B18, 44		19	T.F.
A1, 3, B44, 15, Cw3		3	1	A2, w32, B40, 44, Cw2		13	1
A3, 29, B44, 15, Cw3		3	-2	A2, 11, B15, w41, Cw3		1	2
A11, Bw21, 35, Cw4		4	3	SRBC-rosetted effector cells against autologous target (see text)		34	1
Sens. donor OT†: HLA-Aw24, 26, B27, Cw2							
Targets:							
Autologous		34	3				
Aw24, 25, B27, 40, Cw1, 3		13	2				
Aw24, 26, Bw16		13	5				
A2, Bw35, 40, Cw3		9	2				

Mononuclear cells were isolated by Ficoll-hypaque flotation from defibrinated venous blood. Responder cells from the sensitised donors were resuspended to a concentration of 1.5×10^6 cells ml^{-1} in RPMI-1640 supplemented with antibiotics, glutamine and 15% pooled heat-inactivated human serum ('Ned. H'). Stimulator cells were resuspended in RPMI-1640 ($\approx 5 \times 10^6$ cells ml^{-1}) and to each ml of suspension was added 0.1 μl of a 2% solution of DNFB in acetone, followed by incubation at room temperature in the dark for 30 min. The DNFB-treated cells were washed twice, resuspended in Med. H at a concentration of 1.5×10^6 cells ml^{-1} and irradiated with 2,300 rad. Equal amounts of responder and stimulator cell suspensions were mixed and incubated in flat-bottomed tubes in 5-ml portions for 8 d at 37 °C in a humidified atmosphere with 5% CO_2 . The portions were then pooled, the cells washed once, and resuspended in fresh Med. H at a concentration of 5×10^6 Trypan-blue excluding cells per ml (effector cells). Target cells were prepared by incubating 2×10^6 phytohaemagglutinin (PHA)-stimulated mononuclear cells (1 μl PHA-P (Wellcome) per ml suspension of 1×10^6 cells ml^{-1} in Med. H incubated for 3 d) with 250 μCi $\text{Na}_2^{51}\text{CrO}_3$ in 0.5 ml RPMI for 45 min at 37 °C. Some of the target cells were coupled with DNFB as described above, but five times as much DNFB was used per ml of cell suspension. The washed target cells were resuspended at a concentration of 1×10^6 cells ml^{-1} in Med. H. 200 μl of the effector cell suspension and 100 μl of the target cell suspension were mixed in roundbottomed microtest tubes and incubated for 5 h at 37 °C. The test tubes were centrifuged and radioactivity in the supernatant and cell pellet was counted in a gammacounter. Target cells were incubated in parallel in medium alone and the ^{51}Cr releases were calculated as percentage of ^{51}Cr released relative to the amount incorporated in the target cells at the start of the experiment. Specific ^{51}Cr -release was expressed as (release in mixtures with effector cells) - (release in medium alone). The releases in medium (control) were between 7% and 15%. Experiments were carried out in duplicate or triplicate, and s.e.m.s. were between 1 and 3%. A reaction was considered positive when the specific release exceeded 5%.

* Donors KR, JR and VH were sensitised by injection of autologous DNFB-treated cells.

† Donors OT and MC were sensitised by the application of DNCB on the skin.

TF, Technical failure.

Antigens in common between effector and target cells and italicised. Two cases of target cell killing not restricted by HLA-A, B, C are also italicised.

that the phenomenon of MHC-restricted cell-mediated cytotoxicity also applies to man. We have no unequivocal proof that the cytotoxicity is mediated by T cells. However, more than 90% of the viable cells in the washed effector cell suspension formed rosettes with sheep red blood cells (SRBC). In donor MC an SRBC-rosetted effector cell suspension was separated by Ficoll-Hypaque density flotation. Too few cells were found in the interphase (non-rosetted cells) to be tested for cytotoxic activity. The sedimented SRBC-rosetted cells were collected and after lysis of the SRBC by fresh human AB serum, the cells were tested for cytotoxic activity. These cells had strong DNFB-directed cytotoxic activity (Table 1).

In the three cases where the effector cell donor was HLA-A2, most of the reactivity could be explained by preferential restriction by A2 alone. In fact, the experiments involving this antigen gave the highest specific releases. This might suggest that there is a hierarchy of antigenicity among the antigenic determinants produced by DNFB treatment, in the sense that one HLA antigen conjunction, when available, is preferred to the other possible conjunctions. Preferential restriction by only some of the MHC antigens available has been seen in several mice⁶⁻⁸, and might be

caused either by properties of the MHC antigens themselves or by immune response genes. Whereas the first possibility seems most likely in the present case (particularly the A2 antigen may have a unique role in cytotoxicity), evidence has been presented which evokes Ir genes in the mice systems^{6,7}. Only some of the sensitised donors generated DNFB-directed cytotoxicity detectable by our *in vitro* system. The successful cases could be fortuitous results of a complex multifactorial interaction, but it is possible that a major factor of genetically determined immune responsiveness may have a role. Further studies are needed to clarify these questions, which have obvious implications for our understanding of the maintenance of MHC polymorphism.

The two cases of non HLA-A, B, C restricted target cell killing (if not technical artefacts) could be explained by (1) cytotoxicity exclusively directed against DNFB; (2) DNFB treatment may make target cells liable to killing by cytotoxic effector cells of any specificity; (3) cytotoxicity could be directed against DNFB-'modified' common cell-surface components other than the known HLA-A,B,C antigens; and (4) cross reactions between DNFB-'modified' HLA-A, B, C antigens which are not necessarily revealed by

the known serological cross reactions between HLA antigens. The first two possibilities seem unlikely in view of the many negative reactions with DNFB-treated target cells shown in Table 1.

The DNFB-directed cytotoxic activity could be induced *in vitro* only in lymphocytes from sensitised donors, whereas alloaggressive cytotoxicity is easily induced after primary sensitisation *in vitro*⁹. This presumably reflects the phenomenon emphasised by Simonsen¹⁰, that the number of antigen-sensitive cells primarily reactive against allotypic MHC-antigens in an unsensitised organism is much greater than that of cells primarily reactive against other antigens.

The main purpose of the present study was to investigate whether the phenomenon of MHC-restricted cell-mediated cytotoxicity applies also in man. Having shown that this is the case, work is now in progress to help resolve some of the new questions raised. Moreover, it seems likely that studies of contact hypersensitivity diseases with modifications of the present technique could elucidate the pathogenesis in these disorders.

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E. DICKMEISS
B. SOEBERG
A. SVEJGAARD

Medical Departments P and TA,
Tissue Typing Laboratory,
State University Hospital (Rigshospitalet),
Blegdamsvej 9,
DK-2100 Copenhagen Ø, Denmark

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Conventional calcium channel mediates asynchronous acetylcholine release by motor nerve impulses

STIMULATION of the motor nerve, in addition to producing the synchronous, impulsive release of acetylcholine (ACh) that is recorded electrophysiologically as the endplate potential (e.p.p.)¹, also elicits a delayed, asynchronous discharge of ACh quanta that appears as increases in miniature endplate potential (m.e.p.p.) frequencies^{2–4}. The synchronous release of ACh is mediated by the movement of Ca^{2+} through specific conductance channels activated by depolarisation of the motor nerve ending (for reviews see refs 1, 5 and 6). Although asynchronous release is dependent in some fashion on extracellular (Ca^{2+}) (ref. 4) there seems to be a controversy as to the precise nature of the ionic pathway responsible for this dependence. For example, although it has been suggested that asynchronous evoked release is mediated by residual Ca^{2+} that enters through the traditional Ca^{2+} conductance pathway³, results with Mg^{2+} have suggested the contrary⁴. Specifically, it has been shown that Mg^{2+} , rather than antagonising the asynchronous release of ACh in Ca^{2+} solutions, (as would be expected if Ca^{2+} moved through the same conductance pathway for both forms of release^{7–10}), actually enhanced the asynchronous discharge of ACh quanta⁴. The present study investi-

gated the effects of the conventional Ca^{2+} antagonists, Co^{2+} and Mg^{2+} , on the asynchronous evoked release of ACh. The results demonstrate that after brief, repetitive stimulation, both ions competitively antagonise asynchronous ACh release in a manner similar to their respective antagonism of synchronous release^{7,10}. We conclude that Ca^{2+} supports both dispersed and synchronous ACh release through the same conductance pathway.

Experiments were carried out using isolated nerve-cutaneous pectoris preparations of the frog, which were bathed in normal Ringer solution of the following composition (mM): NaCl 115, KCl 2, NaHCO_3 2 and CaCl_2 1.8 (pH 6.8–7.2). Neostigmine methylsulphate ($1 \mu\text{g ml}^{-1}$) was added to all solutions to increase the size of the m.e.p.ps. In most experiments, after an initial wash in normal Ringer, 400 mM glycerol was added to the normal Ringer (glycerol Ringer) and the preparation bathed in glycerol Ringer for 1 h. When the preparation is returned to normal Ringer after glycerol treatment, the transverse tubule system of the muscle ruptures¹¹. This procedure allowed evoked ACh release to be studied in a full range of Ca^{2+} concentrations without fear of dislodging the recording electrode mechanically from the interior of the muscle fibre. Intracellular recordings were obtained from endplate regions using microelectrodes filled with 3 M KCl (resistances ranging from 10–25 MΩ). The signal from the microelectrode was fed into a conventional preamplifier (W. P. Instruments) and then in parallel into an oscilloscope (Tektronix model 5103N) and a pen recorder (Brush-Gould model 220). M.e.p.p. frequencies were measured from pen records. In the presence of Ca^{2+} , stimuli were delivered to the motor nerve at a frequency of 20 Hz (for 0.5–5 s) and the evoked

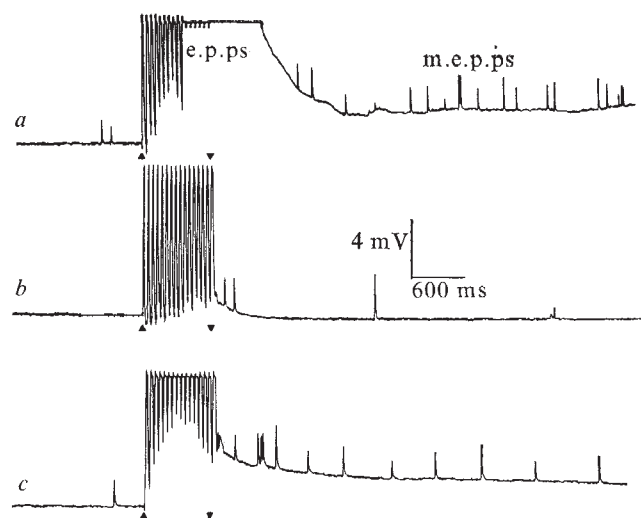


Fig. 1 Competitive antagonism of Ca^{2+} -mediated asynchronous release by Co^{2+} . Stimuli were delivered at 20 Hz for 800 ms (▲). The preparation was pretreated with glycerol Ringer. Evoked m.e.p.p. frequencies were determined during the 4-s period immediately after nerve stimulation. All figures are photographs of pen recorder traces. *a*, Normal (1.8 mM Ca^{2+}) Ringer. During nerve stimulation large e.p.ps are produced (which are superimposed on the underlying depolarisation produced by residual ACh). After nerve stimulation, a residual discharge of individual m.e.p.ps occurs. For *a*, *b* and *c*, resting m.e.p.p. frequency was 2 s^{-1} . Constancy of resting m.e.p.p. frequency was maintained by adding appropriate amounts of sucrose to the Ringer solution. In *a* the m.e.p.p. frequency after stimulation was 4.4 s^{-1} ; *b*, 1.8 mM $\text{Ca}^{2+} + 0.5 \text{ mM Co}^{2+}$ Ringer, note the antagonism of both e.p.p. amplitude and evoked m.e.p.p. discharge by Co^{2+} . M.e.p.p. frequency after stimulation 2 s^{-1} ; *c*, 7.0 mM $\text{Ca}^{2+} + 0.5 \text{ mM Co}^{2+}$ note partial surmounting of Co^{2+} -induced depression by increasing the $[\text{Ca}^{2+}]$. M.e.p.p. frequency after stimulation 3.1 s^{-1} . In this experiment, 9.0 mM $\text{Ca}^{2+} + 0.5 \text{ mM Co}^{2+}$ Ringer produced a matching evoked m.e.p.p. discharge to that produced in normal Ringer.

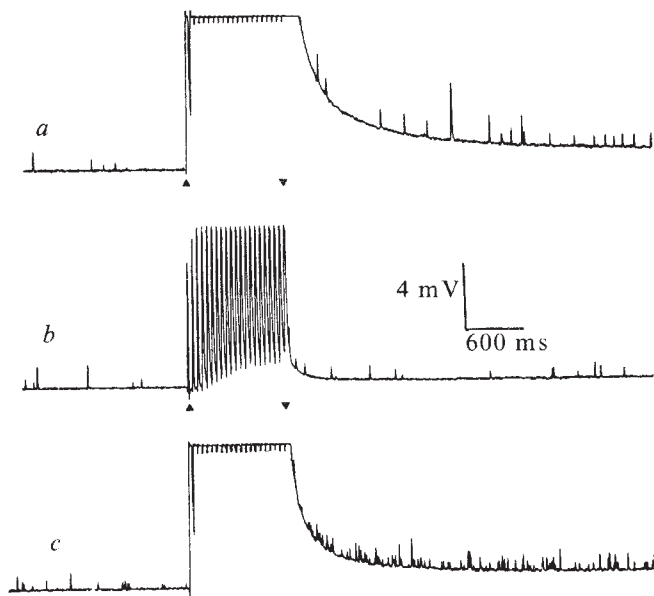


Fig. 2 Antagonism of Ca^{2+} -mediated, asynchronous release by Mg^{2+} . The preparation was pretreated with glycerol Ringer. Stimulation ($\blacktriangle$) was delivered at a frequency of 20 Hz for 1 s. *a*, 1.8 mM Ca^{2+} Ringer resting m.e.p.p. frequency, 2.4 s^{-1} , evoked m.e.p.p. frequency, 5.9 s^{-1} ; *b*, 1.8 mM Ca^{2+} + 10 mM Mg^{2+} Ringer, resting m.e.p.p. frequency, 3.9 s^{-1} , evoked m.e.p.p. frequency, 4.1 s^{-1} , note the antagonism of evoked m.e.p.p.s by Mg^{2+} ; *c*, 6.25 mM Ca^{2+} + 10 mM Mg^{2+} Ringer, resting m.e.p.p. frequency, 5.8 s^{-1} , evoked m.e.p.p., frequency 11.6 s^{-1} . Antagonism was surmounted by increasing the $[\text{Ca}^{2+}]$. For further explanation, see Fig. 1.

m.e.p.p. frequencies measured for the 4-s period after stimulation. During stimulation, a large depolarisation of the postsynaptic membrane (produced by inadequate removal of the enormous quantities of ACh poured into the synaptic cleft¹²) drove the recorder pen off the scale (see, for example, Figs 1 and 2). To ensure faithful counting of m.e.p.p. frequencies during repolarisation after stimulation, either (1), one output of the recorder was differentially biased with respect to the other, so that when the pen had moved off the scale on the lower part of one channel, it appeared on the scale at the upper part of the other channel or (2), the output terminal of the a.c.-coupled oscilloscope amplifier was fed directly into the recorder.

Figure 1 illustrates a typical experimental result from a preparation pretreated with glycerol Ringer. In Fig. 1*a*, repetitive nerve stimulation in normal Ringer (1.8 mM Ca^{2+}) produced e.p.p.s superimposed on a depolarising membrane potential and a residual discharge of m.e.p.p.s after the stimulation period. In this trace, the m.e.p.p. frequency increased from a resting level of 2 s^{-1} to approximately 4.5 s^{-1} after stimulation. The addition of 0.5 mM Co^{2+} to normal Ringer (Fig. 1*b*) depressed the e.p.p. amplitude and fully antagonised the evoked discharge of m.e.p.p.s. Increasing the extracellular Ca^{2+} to 7.0 mM in the presence of Co^{2+} (Fig. 1*c*) enabled the antagonism of both the e.p.p. amplitude and evoked m.e.p.p. frequency to be overcome. In this experiment, a concentration of 9.0 mM Ca^{2+} was necessary to produce an evoked discharge of m.e.p.p.s that matched the control response (Fig. 1*a*). Note that in this experiment, changes in the resting m.e.p.p. frequencies (as a consequence of tonicity changes of the various Ringer solutions) were prevented by the addition of appropriate amounts of sucrose to the Ringer. In several experiments it was possible to construct a log dose-response curve for Ca^{2+} -evoked m.e.p.p.s and to observe a parallel shift in this curve in the presence of Co^{2+} . This suggests that Co^{2+} acts as a competitive antagonist of asynchronous

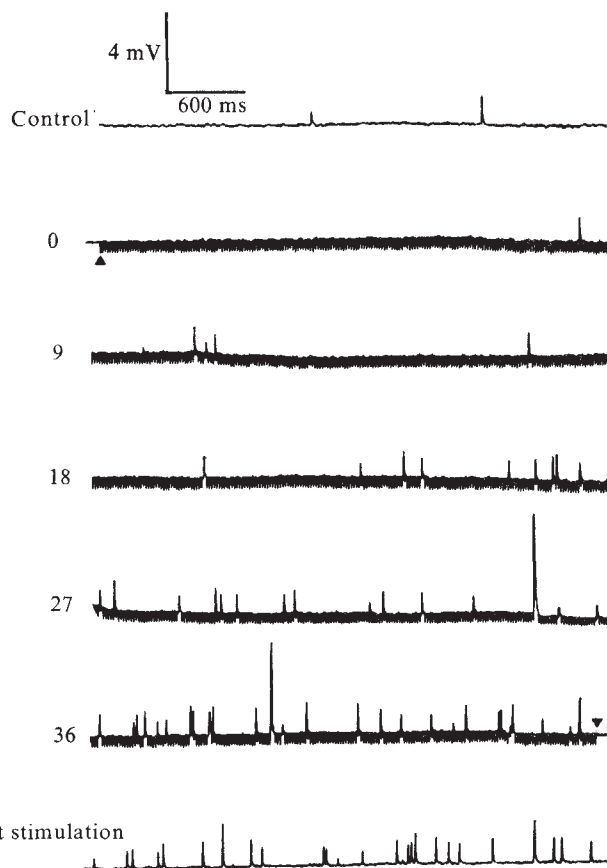
release^{8,12}, much as it does for synchronous release^{10,13}. The equilibrium dissociation constant (K_d) for Co^{2+} as a competitive antagonist of asynchronous release can be calculated as follows^{8,13}

$$K_d = ((\text{Ca}_2/\text{Ca}_1) - 1)^{-1} [\text{antagonist}] \quad (1)$$

where Ca_1 is the $[\text{Ca}^{2+}]$ that produces a certain evoked discharge of m.e.p.p.s and Ca_2 is the $[\text{Ca}^{2+}]$ that produces an identical evoked discharge of m.e.p.p.s in the presence of the antagonist. In four experiments in which the resting m.e.p.p. frequency remained essentially unchanged, the K_d for Co^{2+} ranged from 0.10 to 0.15 mM with a mean for all experiments of 0.13 mM. This value compares favourably with the reported values of Co^{2+} as an antagonist of synchronous release (0.07 mM (ref. 10), 0.18 mM (ref. 14)) suggesting that both forms of release may share a common conductance pathway.

Further evidence in support of this contention was provided by experiments using Mg^{2+} . In the experiment shown in Fig. 2, stimulation in normal Ringer after glycerol treatment produced a large increase in m.e.p.p. frequency from a resting level of 2.4 s^{-1} to 6 s^{-1} after stimulation. Addition of 10 mM Mg^{2+} , although causing a slight increase in resting frequency to 3.9 s^{-1} (presumably because of increased tonicity of the Ringer), fully antagonised the evoked discharge of m.e.p.p.s. Increasing the $[\text{Ca}^{2+}]$ to 6.25 mM,

Fig. 3 Slow increases in m.e.p.p. frequency evoked by prolonged (40 s) stimulation at 50 Hz in 10 mM Mg^{2+} Ringer. Upper trace, control m.e.p.p. frequency, 0.5 s^{-1} ; Middle traces, m.e.p.p. frequencies at indicated times after starting stimulation. Spikes on baseline indicates stimulus artefacts. Lowest trace, m.e.p.p. frequency immediately after stimulation, 6 s^{-1} . Note that a discernible increase in m.e.p.p. frequency begins only after 18 s of stimulation. Similar records were produced in the presence of 1 mM Co^{2+} .



although further increasing the resting frequency, overcame the antagonism of evoked m.e.p.s by Mg^{2+} . Experiments where the change in resting m.e.p.p. frequency was prevented by adding sucrose to the Ringer solutions were used to calculate the K_d for Mg^{2+} (equation 1). Indeed, the mean K_d for Mg^{2+} is 4.6 ± 0.2 mM (mean $\pm$ s.e., $n=4$) as an antagonist of asynchronous release is similar to the values for Mg^{2+} as an antagonist of synchronous release (4.0 mM, 3.0 mM, 4.4 mM: refs 8–10 respectively).

These results, demonstrating that Mg^{2+} antagonises the asynchronous Ca^{2+} -dependent discharge of m.e.p.s seem to be at variance with those suggesting that Mg^{2+} enhances the asynchronous release of ACh. It is possible that the ability of Mg^{2+} to activate asynchronous evoked release may appear only after prolonged, high frequency stimulation. Figure 3 illustrates a representative experiment which suggests that this is the case. Note that it is only after 18 s of high frequency (50 Hz) stimulation in 10 mM Mg^{2+} Ringer that discernable increases in m.e.p.p. frequency were observed. This is in contrast to the results shown in Figs 1 and 2, where stimuli were delivered at a frequency of 20 Hz for a brief period (≤ 1 s). In the experiment of Fig. 3 m.e.p.p. frequencies were elevated from the control level of $0.5 s^{-1}$ (upper trace) to $6 s^{-1}$ immediately after 40 s of stimulation (lowest trace). A concentration of Co^{2+} as high as 1 mM did not alter either the time course or the absolute increase in m.e.p.p. frequency produced by stimulation in 10 mM Mg^{2+} Ringer. It thus seems that Ca^{2+} and Mg^{2+} support asynchronous release through different ionic conductance channels^{4,13}.

In conclusion, the results suggest that the Ca^{2+} -dependent asynchronous release of ACh by nerve stimulation is mediated by the same conductance pathway as synchronous release. It is interesting that Ba^{2+} (which does not support synchronous release¹⁶) and Sr^{2+} (which can only poorly support synchronous release¹⁷) both produce large discharges of m.e.p.s after brief repetitive nerve stimulation. In contrast to Mg^{2+} , however, both Ba^{2+} and Sr^{2+} support asynchronous release through the same conductance pathway normally traversed by Ca^{2+} in mediating the synchronous and asynchronous discharge of ACh quanta (ref. 18 and our work in preparation).

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E. M. SILINSKY
A. M. MELLOW
T. E. PHILLIPS

Laboratory of Presynaptic Events,
Department of Pharmacology,
School of Medicine,
Northwestern University,
Chicago, Illinois 60611

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Role of prostaglandin-mediated vasodilatation in inflammation

INFLAMMATORY reactions are characterised by hyperaemia, and exudation of plasma resulting in tissue swelling. Putative mediators of inflammation have usually been evaluated according to their ability to mimic inflammatory reactions¹. Although prostaglandins were identified in inflammatory exudates², and prostaglandin synthesis *in vitro* was shown to be inhibited by non-steroid anti-inflammatory compounds^{3–5}, prostaglandins (unlike histamine and bradykinin) were found to be poor at eliciting plasma exudation when injected into guinea pig^{6,7} or rabbit skin⁸. But, the finding that exogenous prostaglandins (notably of the E-type) potentiate plasma exudation produced by other mediators^{7–11} suggested that this may be the role of prostaglandins in inflammation. An alternative view has been proposed by Kuehl *et al.*¹², who consider that since E and F prostaglandins fail fully to mimic inflammatory reactions, other products of arachidonic acid should be considered as mediators. They suggest that the unstable prostaglandin endoperoxide, PGG₂, or a non-prostaglandin product of it (a free radical could be the important mediator. We have re-examined the mode of action of prostaglandins in inflammation by measuring both increased blood flow and plasma exudation. The results presented here

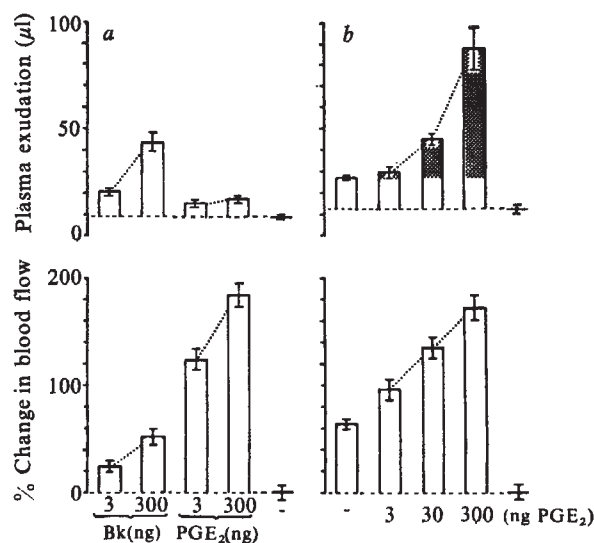


Fig. 1. Plasma exudation (upper) and local blood flow changes (lower) measured simultaneously in rabbit skin in response to various agents. In each experiment a New Zealand White rabbit was given an intravenous injection (approximately 15 μ Ci per kg body weight) of ^{131}I -labelled human serum albumin and a short-acting anaesthetic, methohexitone sodium (approximately 10 mg per kg). Intradermal injections (0.1 ml) of inflammatory agents mixed with ^{133}Xe (5–10 μ Ci per injection) were then given into the clipped back skin in random-block order. After 20 min the animal was killed, skinned, and a 16-mm diameter punch was used to remove injection areas. Samples of skin, blood, and injection fluid were counted in an automatic γ -counter. Plasma volumes of skin samples (μ l per sample) and local blood changes (% increase above controls) were computed as previously described¹⁶. *a*, Comparison of the responses produced by bradykinin (Bk) and PGE₂ at the doses shown. Bradykinin produced exudation with little blood flow increase. Conversely, PGE₂ produced little exudation but a large increase in blood flow. *b*, Effect of adding increasing doses of PGE₂ to a fixed dose of histamine (H, 2.5 μ g) resulting in an increase in blood flow and a potentiation of exudation. In both *a* and *b* the shaded areas denote the amount of exudation produced above that produced by the permeability-increasing agent alone; dashed lines represent saline control level for both plasma volume and blood flow; single dash represents saline injection. Values are means $\pm$ s.e.m., $n = 6$.

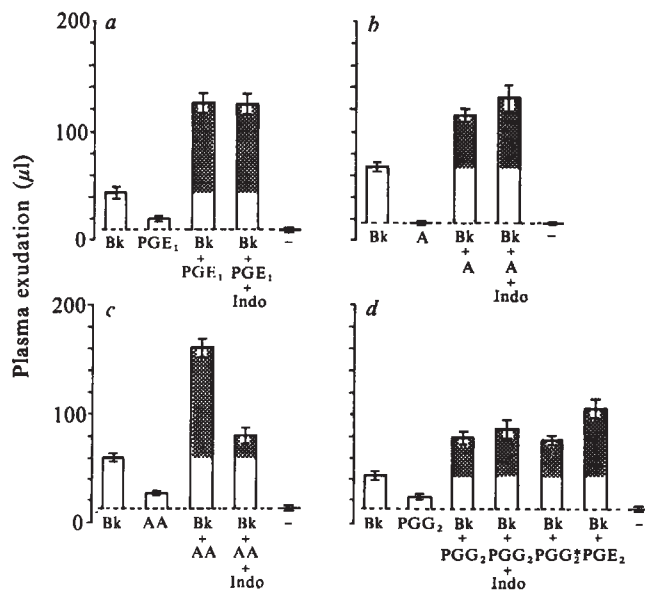


Fig. 2 Four experiments showing potentiation of exudation responses to bradykinin (Bk, 500 ng) by: PGE₁ (10 ng); adenosine (A, 100 μg); arachidonate (AA, 1 μg); and PGG₂ (100 ng). Addition of 1 μg indomethacin (Indo) suppressed only the potentiation produced by arachidonate. Incubation of PGG₂ in saline at 37°C for 10 min (PGG₂*) did not reduce its potentiating activity. Potentiation produced by the same dose (100 ng) of PGE₂ is shown for comparison. Values are means $\pm$ s.e.m., $n = 6$.

suggest that the mediation of vasodilatation and increased vascular permeability should be considered separately. Prostaglandins (notably the E-type^{13,14}) mediate vasodilatation (although they may have some additional mast cell degranulating activity in the rat¹⁵). It is this vasodilatation which is responsible for the potentiation of the exudation produced by other mediators. Our observations provide a new hypothesis for the microvascular mechanisms involved in the action of non-steroid anti-inflammatory compounds.

Plasma exudation and local blood flow changes were measured in rabbit skin using the accumulation of intravenously-injected ¹³¹I-albumin and wash-out of intradermally-injected ¹³³Xe, as previously described¹⁶.

Figure 1a shows that intradermal injection of E-type prostaglandin produced a large increase in local blood flow with little plasma exudation (often undetectable). Bradykinin (and histamine at larger doses), on the other hand, increased vascular permeability resulting in plasma exudation (for 10–15 min following injection¹⁷), but they were far less potent at increasing blood flow. An example of the exudation-potentiating activity of prostaglandins is shown in Fig. 1b, which demonstrates that addition of increasing doses of PGE₂ to a fixed dose of histamine resulted in increasing blood flow and an enhancement of exudation. We have previously reported⁸ that exudation potentiation correlates with the vasodilator activity of prostaglandins, and a similar relationship has been observed using other techniques¹⁸. First, the rank order of different prostaglandins was the same for exudation-potentiality as for blood flow-increasing potency⁸, that is, PGE > PGA > PGF > PGD. Second, local injection of substances, other than prostaglandins, which increased skin blood flow, for example isoprenaline, ADP, and adenosine (see Fig. 2b), also had exudation-potentiating activity⁸. Conversely, substances which reduced skin blood flow, for example noradrenaline and angiotensin II, reduced exudation and blood flow in parallel⁸. From these results we conclude that prostaglandins increase histamine- and bradykinin-induced plasma exudation not by increasing vascular permeability, but by increasing vasodilatation. The exact mechanism of this is not clear, but extrapolation from observations on the transport of

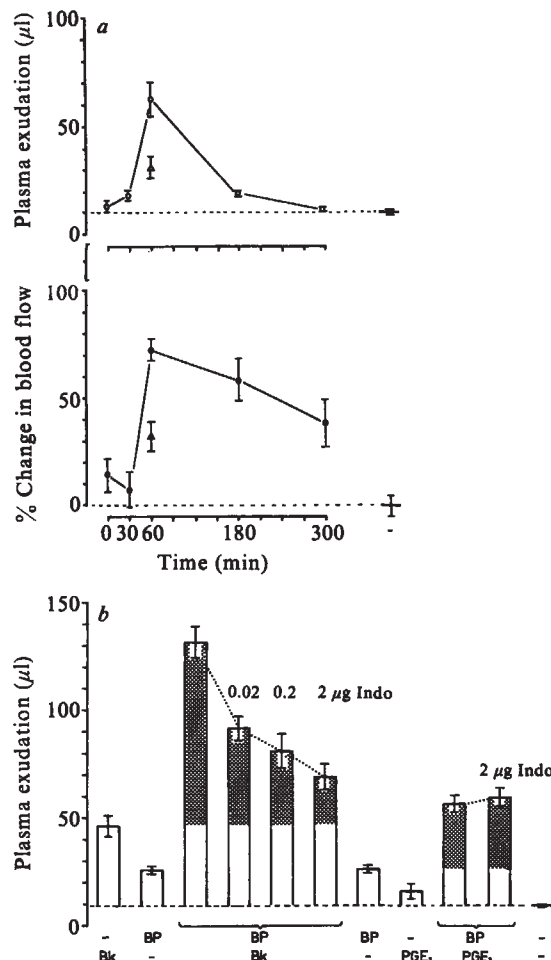


Fig. 3 a, Time course of exudation ($\circ$), and blood flow changes ($\bullet$) produced by intradermal injection of killed *B. pertussis* vaccine (2.5×10^8 organisms per site) into an unsensitized rabbit. Responses varied between animals; for example exudation, mean $23.1 \pm 3.0 \mu\text{l}$, range 3.6–71.9 μl , $n = 32$ rabbits (six measurements per animal). Abscissa is time between *B. pertussis* injections and following injections of isotopes. Exudation and blood flow were measured over a further 20 min period. Indomethacin (1 μg), mixed with the vaccine before injection reduced both blood flow increase ($\blacktriangle$) and, therefore (according to the hypothesis), exudation (Δ). b, Enhanced exudation, at the peak of the response to intradermal *B. pertussis* injection, produced by a further injection of either bradykinin or PGE₁. Potentiation of exudation produced by a first injection (scale top line) of *B. pertussis* (BP) and a second injection (lower line), 60 min later, of bradykinin (500 ng). Exudation was measured over the following 30 min. This effect was suppressed by locally-injected indomethacin (dose equally divided between first and second injections). The potentiation produced by a first injection of *B. pertussis* and a second injection of PGE₁ (500 ng) was unaffected by locally-injected indomethacin. Values are means $\pm$ s.e.m., $n = 6$.

macromolecules from blood to lymph in dog paw following thermal injury¹⁹, suggest that exudation potentiation results from an increase in total vessel wall exchange area in the dilated vascular bed.

Figure 2 shows potentiation of bradykinin-induced exudation by PGE₁, adenosine, arachidonate²⁰, and PGG₂. Adenosine is included as an example of a non-prostaglandin vasodilator. All four agents produced little exudation when injected alone. Potentiation produced by PGE₁ (and PGE₂, not shown), adenosine, and PGG₂, was unaffected by locally-injected indomethacin; whereas that produced by arachidonate, the PGE₂ precursor (and similarly dihomo- γ -linolenate, the PGE₁ precursor) was suppressed. These results are consistent with the proposition that indomethacin suppresses inflammatory dilatation, and as a consequence inflammatory oedema, by preventing the production of a vasodilator substance via the cyclo-oxygenase pathway. Although the importance of the unstable prostaglandin endoperoxide, PGG₂, has been emphasised¹², it is interesting to

note that PGG_2 injected alone produced little exudation. Furthermore, the exudation-potentiating potency of PGG_2 was less than that of PGE_2 and was unaffected by incubation in saline for 10 min at 37°C (which would leave little PGG_2 unchanged). This would suggest that the observed activity of PGG_2 *in vivo* was due to one of its products, PGE_2 . Indeed, although injection of PGG_2 produced a net increase in blood flow over the period measured (20 min), other evidence based on the time course of ^{133}Xe clearance²¹ would suggest that the endoperoxides themselves may have vasoconstrictor activity.

The results obtained using exogenous agents suggest that inflammation may involve the separate production of permeability-increasing mediators and vasodilator mediators. This is supported by results from experiments using intradermal injections of killed *Bordetella pertussis* vaccine in unsensitized rabbits. Other materials produced similar results, for example Zymosan, glycogen and carrageenan. Intradermal injection of *B. pertussis* resulted in exudation and increased blood flow with a peak at 60–80 min (Fig. 3a). Indomethacin suppressed both exudation and increased blood flow (Fig. 3a). The presence of an endogenous vasodilator (exudation-potentiating) mediator was established by injecting bradykinin at the peak of the response, 60 min after *B. pertussis* injection. The exudation response to bradykinin was potentiated (Fig. 3b) and this effect was inhibited by locally-injected indomethacin. The presence of an endogenous permeability-increasing mediator (unlikely to be either histamine or a kinin; T.J.W., unpublished observations) was established by injection of PGE_1 60 min after *B. pertussis* injection. PGE_1 also produced potentiation (Fig. 3b) but this effect was unaffected by indomethacin. Thus, indomethacin inhibited the production of the vasodilator (exudation-potentiating) mediator(s), and not the production of the permeability-increasing mediator(s).

We conclude that vascular changes in inflammatory reactions should be considered in terms of two types of chemical mediators—mediators which are predominantly vasodilators and mediators which are important for their vascular permeability-increasing activity. The amount of plasma exudation is dependent on the level of both types of mediators; and it follows that oedema can be suppressed by inhibiting the production, or action, of either type. Permeability-increasing mediators seem to be independent of the cyclo-oxygenase pathway of arachidonate (or dihomogamma-linolenate) metabolism. No evidence was obtained in support of the recently suggested¹² pivotal role for PGG_2 ; in inflammation the important substances derived from arachidonate (or dihomogamma-linolenate) are vasodilators, probably E-type prostaglandins (although the recently discovered PGI_2 cannot be excluded from a similar role). Indomethacin was found to inhibit specifically the production of vasodilator mediators. Thus, we propose that non-steroid anti-inflammatory compounds reduce inflammatory oedema as a consequence of a suppression of vasodilatation.

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T. J. WILLIAMS
M. J. PECK

Department of Pharmacology,
Institute of Basic Medical Sciences,
Royal College of Surgeons of England,
Lincoln's Inn Fields,
London WC2, UK

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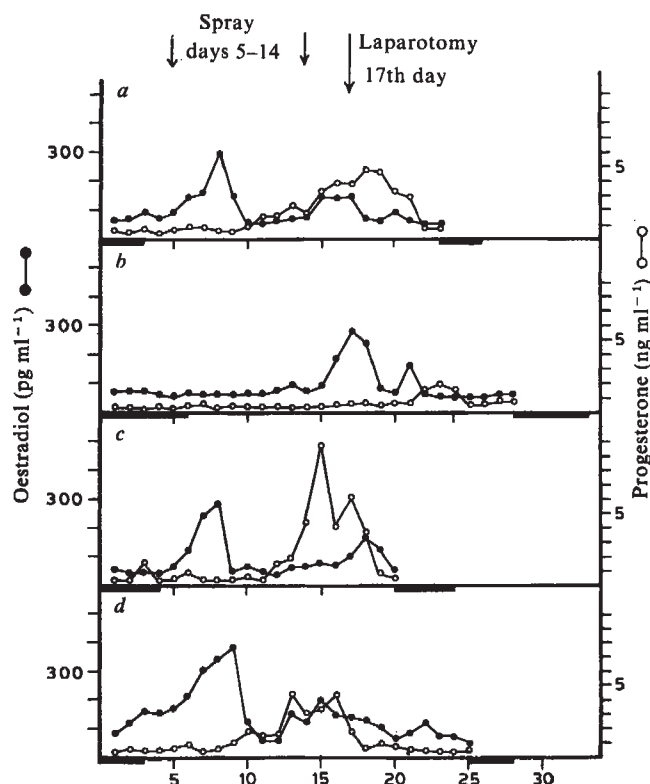
Ovulation in rhesus monkeys suppressed by intranasal administration of progesterone and norethisterone

THE importance of various brain structures in the regulation of secretion of gonadotropins^{1–3} and the presence of steroid hormones in the cerebrospinal fluid (CSF)^{4–5} suggest that a method for delivering steroids into the CSF might be useful in the control of fertility. Steroids can enter the cerebral components rapidly after intranasal administration and their concentrations are higher than after intravenous injection⁶. We report here that progesterone and norethisterone given intranasally can prevent ovulation in rhesus monkeys.

Our studies were carried out between September and March during when almost all the monkeys in our colonies have ovulatory menstrual cycles. We used adult female rhesus monkeys (5–6 kg) which had experienced at least two successive ovulatory menstrual cycles of normal duration (20–33 d) (determined by monitoring circulating levels of progesterone throughout the menstrual cycles).

Two steroids, progesterone, a naturally occurring ovarian hormone and norethisterone, a synthetic progestogen widely used in hormonal contraception were

Fig. 1 Effects of spraying progesterone intranasally in rhesus monkeys. Serum profiles of oestradiol and progesterone throughout the menstrual cycle are shown for one monkey representative of each of the four treated groups. Ovulation was suppressed only in the monkey given $2\mu\text{g d}^{-1}$. Black bars on x axis indicate menstruation. a, Control monkey no. 388; b, monkey no. 695, $2\mu\text{g d}^{-1}$; c, monkey no. 690, $10\mu\text{g d}^{-1}$; d, monkey no. 699, $30\mu\text{g d}^{-1}$.



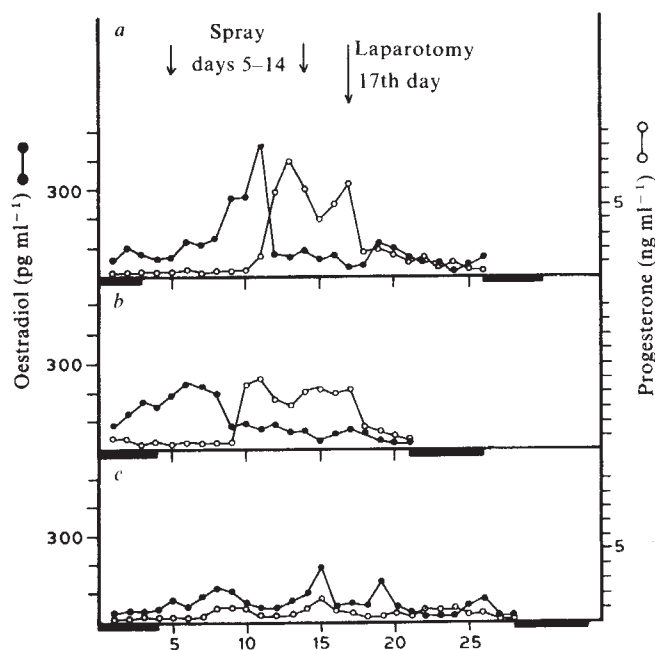


Fig. 2 Effects of spraying norethisterone intranasally in rhesus monkeys. Serum profiles of oestradiol and progesterone throughout the menstrual cycle are shown for one monkey representative of each of the three treated groups. Ovulation was suppressed only in the monkey given $9 \mu\text{g d}^{-1}$. Black bars on x axis indicate menstruation. *a*, Control monkey no. 705; *b*, monkey no. 703, $3 \mu\text{g d}^{-1}$; *c*, monkey no. 695, $9 \mu\text{g d}^{-1}$.

dissolved in a mixture of ethanol:propylene glycol:water (1:1:3). A constant volume (0.5 ml) of this mixture was put into a glass atomiser (Brovon Inhaler, Moore Medicinal Products Ltd), which was inserted into one nostril of a conscious monkey seated on a restraining chair. The mixture was sprayed into the nostril for 10 s by connecting the atomiser to a nitrogen source regulated to deliver the inert gas at a constant pressure of 0.25 kg cm^{-2} . Before use, each spraying device was calibrated to deliver the drug for 10 s. The amount of steroid administered to each animal was regulated either by varying the concentration of the steroid in the solvent or by repeated spraying for periods of 10 s. Controls were sprayed with the solvent alone.

The effects on ovulation were determined by measuring serum levels of oestradiol and progesterone in blood samples taken daily throughout the menstrual cycle, as well as by visual observation of the ovaries after laparotomy on day 17 of the menstrual cycle. Circulating levels of progesterone below 2 ng ml^{-1} serum together with the regressed state of ovaries at laparotomy were taken as indicative that ovulation had been suppressed. It was assumed to have occurred when circulating levels of progesterone were greater than 3 ng ml^{-1} serum and up to $5\text{--}6 \text{ ng ml}^{-1}$, and when at the same time one of the ovaries was enlarged and had a newly formed corpus luteum.

Among monkeys treated with progesterone, ovulation was always suppressed by $2 \mu\text{g d}^{-1}$ and also in two out of six cases when $10 \mu\text{g d}^{-1}$ was given. Ovulation occurred in four of the six monkeys given $10 \mu\text{g d}^{-1}$ as well as in all those given $30 \mu\text{g d}^{-1}$ (Table 1, Fig. 1).

In the monkeys treated with norethisterone, ovulation was unaffected by $3 \mu\text{g d}^{-1}$ but was suppressed in all cases when 9 or $17 \mu\text{g d}^{-1}$ was given (Table 1, Fig. 2).

These results show that intranasal administration of steroids can suppress ovulation. The two steroids had different effects: progesterone suppressed ovulation at the lowest dose but not in all animals at higher doses. Norethisterone was effective only at the two higher doses. The

Table 1 Effect of spraying steroid hormones intranasally during days 5–14 of menstrual cycle

Treatment	Monkey no.	Ovulation	Menstrual cycle length (d)	
			Untreated	Treated
Controls	388	+	31	23
	630	+	25	25
	687	+	30	30
	694	+	24	23
	705*	+	26	26
	717*	+	24	23
Progesterone	673	—	27	21
	688	—	22	29
	DD, $2 \mu\text{g}$	—	23	27
	TD, $20 \mu\text{g}$	—	26	29
	704	—	25	33
	727*	—	28	31
Group B	655	—	29	23
	DD, $10 \mu\text{g}$	+	24	20
	TD, $100 \mu\text{g}$	+	25	24
	695*	+	31	22
	696*	+	30	50
	700	—	33	27
Group C	699	+	25	21
	DD, $30 \mu\text{g}$	+	26	30
	TD, $300 \mu\text{g}$	+	23	26
Norethisterone Group D	728	+	23	26
	703*	+	24	21
Group E	DD, $9 \mu\text{g}$	—	26	28
	TD, $90 \mu\text{g}$	—	24	28
	705*	—	26	28
Group E	DD, $17 \mu\text{g}$	—	21	113
	TD, $170 \mu\text{g}$	—	23	65

Controls were sprayed with solvent only. DD, daily dose; TD, total dose; +, occurrence of ovulation; —, no ovulation.

*At least two successive ovulatory menstrual cycles of normal duration were observed in those animals before they were used in their experiments.

different effects of progesterone is not surprising in view of its ability to inhibit or facilitate gonadotropin secretion depending on circumstances. When administered to monkeys after oestradiol, it inhibits the oestrogen-induced surge of luteinising hormone (LH)⁷; if given in conjunction with oestradiol, it significantly advances this surge⁸. A facilitatory effect of progesterone on LH secretion has also been demonstrated in women^{9,10}. In contrast, there is evidence that progesterone inhibits gonadotropin secretion and thus blocks ovulation in monkeys^{7,11} and women¹².

Further work is needed to explain the two dose-related, pharmacological effects of progesterone which we have observed.

T. C. ANAND KUMAR
G. F. X. DAVID
V. PURI

Neuroendocrine Research Laboratory,
Department of Anatomy,
WHO Collaborating Centre for Research
and Training in Human Reproduction,
All India Institute of Medical Sciences,
New Delhi—110 016, India

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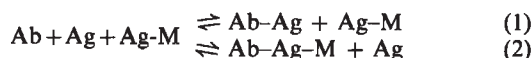
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Metalloimmunoassay

THE disadvantages encountered with radioisotopes, such as high costs, health hazards, limited variety of useable isotopes, short shelf-life of the labelled antigens and difficulties of introducing the radioactive isotope on to the antigen molecule, have stimulated the search for non-isotopic methods in immunological work¹. We report here on the feasibility of a novel non-isotopic system for immunochemical studies and its possible use in immunoassays. The basic concept of the new system is the use of metal atoms in the form of their organometallic or co-ordination complexes as labelling agents for haptens or macromolecular antigens.

The extensive chemistry of transition metal-organic complexes developed during the past two decades should provide a rich and versatile source for the synthesis of 'tailor-made' metal-labelled antigens, which would be free of the disadvantages of radio isotopes. The metal-labelled antigens are designated metallo-antigens and/or metallohapten and we suggest that the immunoassay based on these reagents be called metalloimmunoassay (MIA).

The basic requirements for a metalloimmunoassay are (1) preparation of reagents (antibodies and metallohapten) and (2) development of a method for quantifying the metal content. The metallohapten (Ag-M) and the unlabelled antigen (Ag) react with the specific antibodies, according to the competitive protein-binding equations (1) and (2).



After separation of the free, unbound antigen (Ag-M, Ag) from the antibody-antigen complexes (Ab-Ag-M, Ab-Ag), the amount of metal present in the bound (B) or free phase (F) can be determined by suitable analytical methods such as emission, absorption and fluorescence spectrometry, electrochemical methods and neutron activation. Preparation of a calibration curve plotted for standardised amounts of metallohapten and unlabelled antigen provides the means of determining the quantity of analysed substance in unknown samples.

Typical examples of reagent preparations performed during development of a metalloimmunoassay are illustrated in Fig. 1. The oestrogen steroids, oestrone (Ia), oestradiol-17 β (Ib) and oestrinol-16 α , 17 β (Ic) were transformed in high yields (>80%) into their respective 3-O-carboxymethyl derivatives (IIa-c), as well as into oestradiol-17 β hemisuccinate (IVb) and oestrinol-16 α , 17 β -bis-hemisuccinate (IVc). These carboxylic acid derivatives were used for the preparation of bovine serum albumin (BSA) conjugates. The production of antisera (in rabbits) and determination of their titre and specificity by free radical immunoassay² were carried out as described previously³. The antibodies were insolubilised by reaction with cyanogen bromide-activated Sepharose 4B (Pharmacia)⁴ in order to provide a convenient method for separation of free, unbound Ag-M from the antibody-antigen complex.

The same carboxylic acid derivatives (IIa-c and IVb-c) as well as the amino derivatives III and IVa were labelled with various metal complexes to form the oestrogen-metallohapten some of which are shown in Fig. 2. Also shown are examples of cannabinoid and barbiturate metallohapten. The structures of the compounds

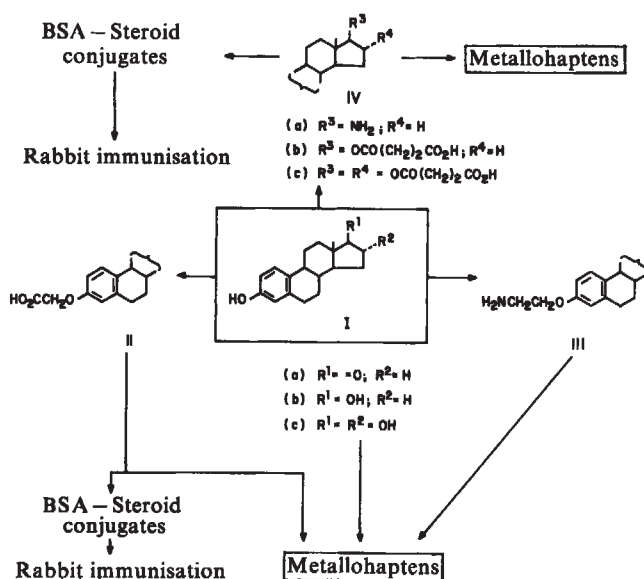
reported here were characterised by routine analytical procedures. BSA, as a representative protein molecule, has been labelled with both iron and manganese complexes, in a 1:20 molar ratio BSA:metal complex.

Atomic absorption spectrometry⁵ was used for detection and quantification of metal concentrations in solutions containing metallohapten. The use of flameless-operation instruments (Perkin Elmer 403 with HGA-70 graphite furnace and Instrumentation 351 with 455 flameless unit and programmable calculation data system kit 42168-02) enabled us to obtain satisfactory calibration curves in the metal concentration range 2-50 ng ml⁻¹. This corresponds to unlabelled hapten concentrations in the range 20-500 ng ml⁻¹, since the metal content is on the average about 10-20% of the molecular weight of the metallohapten. This concentration range is suitable for the detection of a relatively large number of urinary metabolites such as oestriol (in pregnancy urines), morphine, barbiturates, amphetamine and cocaine, without previous extraction and concentration operations. But, in this early stage of development and given the currently commercially available atomic absorption spectrometers, MIA cannot be used for the detection of picogram hapten concentrations, as can be achieved with radioimmunoassays⁶. Further work may improve detection limits.

Some indication of methodology can be obtained from a brief description of one of the procedures using reagents mentioned above. Standardised solutions of the iron-containing oestradiol-17 β -succinate metallohapten, VII, in 0.01 M phosphate buffer (pH 7.3) with 15% dimethylformamide, yielded an atomic absorption calibration curve, in the 0-50 ng Fe ml⁻¹ concentration range, with a linear regression equation $y = 1.731x + 42.88$ and a correlation coefficient $r = 0.9977$. A Sepharose-bound anti-oestradiol-17 β hemisuccinate antibody preparation was titrated with standard solutions of metallohapten, VII, Vortex-mixed, incubated (30 min at room temperature) and centrifuged (1 min at 3,000 r.p.m.).

Aliquots (20-30 μ l) from the supernatant were injected into the graphite furnace of the atomic absorption spectrometer to measure the amount of free metallohapten, Ag-M. To determine inhibition (%) of metallohapten binding, a similar experiment was carried out using a constant concentration of metallohapten VII and increasing added amounts of unlabelled steroid IVb. With the

Fig. 1 Outline for synthesis of oestrogen haptens, their protein conjugates and metallohapten.



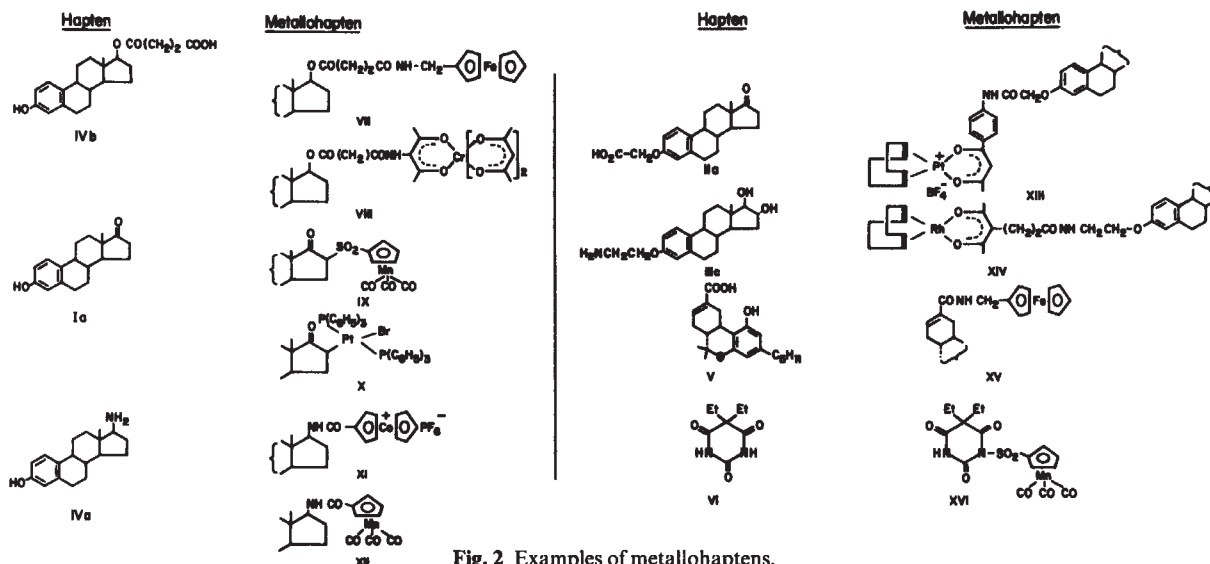


Fig. 2 Examples of metallohapten.

antisera used in this experiment and hapten concentrations of $0.1-1 \mu\text{g ml}^{-1}$, a molar ratio unlabelled steroid to metallohapten of 1.31:1 was required to obtain about 44% inhibition of metallohapten binding. These and similar data demonstrate the feasibility of the concept and preliminary efforts to optimise the assay conditions have been promising.

MICHAEL CAIS*
S. DANI
Y. EDEN
O. GANDOLFI
M. HORN
E. E. ISAACS
Y. JOSEPHY
Y. SAAR
E. SLOVIN
L. SNARSKY

Department of Chemistry,
Technion-Israel Institute of Technology,
Haifa, Israel

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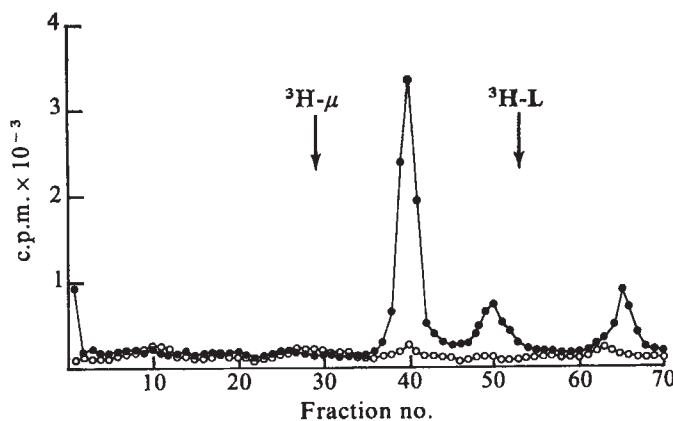
*To whom correspondence should be addressed.

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MW¹. The MHC was originally discovered in the mouse², where it is referred to as the histocompatibility-2 or H-2 system³. Later, similar systems were described in man, rhesus monkey, chimpanzee, rat, dog, rabbit and guinea pig; and, in fact, may exist in most, if not all, mammalian species⁴. Furthermore, clusters of genes controlling graft rejection, mixed lymphocyte reaction (MLR), immune responsiveness, and complement activity have also been reported in the chicken⁵ and *Xenopus*⁶. It is tempting to speculate that these gene clusters controlling immune functions form a single evolutionary line from more primitive ancestral genes in lower vertebrates to the HLA complex in man and the H-2 complex in the mouse⁷. Proof of such a relationship exists for the MHC of man and mouse, where the H-2 and HLA systems show considerable amino acid sequence homology in both classes of molecules⁷. Because of the relative closeness of the two species (H-2 and HLA), homology is not surprising. We have investigated whether the homology extends to other vertebrate classes, such as birds: for example, is the chicken B system a true evolutionary homologue of H-2 or HLA?

Molecules isolated with the use of anti-B sera from chicken lymphocytes were used for amino acid sequence

Fig. 1 SDS-PAGE of ¹²⁵I-labelled B antigen precipitated from lysates of radioiodinated chicken splenocytes. Precipitates were dissolved, reduced, and electrophoresed for 16 h at 4 mA on 7.5% gels together with ³H-μ and L chains. In plotting gel patterns, the ³H markers were aligned. ●, Anti-B; ○, control.



Homology of (murine) H-2 and (human) HLA with a chicken histocompatibility antigen

THE major histocompatibility complex (MHC) is a cluster of genes controlling a variety of immunological and non-immunological phenomena¹. Membrane-bound glycoprotein products of the MHC genes are of two classes: molecules of one class consist of 44,000 MW polypeptide chain which is non-covalently associated with a smaller (12,000) chain of β_2 -microglobulin¹; molecules of the second class consist of two polypeptide chains of 33,000 and 28,000

		5	10	15	20	25	
CHICKEN B	HIS	ARG TYR PHE	ARG	MET		TYR	TYR
MOUSE H-2K ^k	PRO HIS	SER LEU ARG TYR PHE	HIS THR ALA VAL SER ARG PRO	LEU	LYS PRO ARG PHE		TYR
H-2K ^b	PRO HIS	SER LEU ARG TYR PHE	VAL THR ALA VAL SER ARG PRO	LEU	LYS PRO ARG TYR		TYR
HUMAN A ₂	GLY SER HIS	SER MET ARG TYR PHE	PHE THR SER VAL SER ARG PRO GLY	GLY GLY SER ASX PHE ILE ALA VAL			
B ₇	GLY SER	SER MET ARG TYR PHE	TYR THR SER VAL SER ARG PRO GLY	GLY GLU	PHE ILE	VAL	
GUINEA PIG GPI A	HIS	LEU ARG TYR PHE	TYR	ALA VAL	PRO	PHE VAL	TYR

Fig. 2 Comparison of the N-terminal sequences of murine H-2K^k, H-2K^b, guinea pig GPLA, and human HLA-2 and HLA-7 with the chicken B molecule. Residues which show homologies are boxed.

analysis. White Leghorn chickens of the SC strain (Hyline SC; B²B²), 1–4 months old, were killed, spleen cell suspension prepared⁸, and 0.5×10^6 – 3×10^6 cells labelled with single amino acids as described previously⁸. Cells were lysed in 0.5% Nonidet P40 (NP40) (Gallard Schlesinger) (10^6 nucleated cells ml⁻¹) and the lysates centrifuged and dialysed for 16–24 h at 4 °C against phosphate-buffered saline (PBS) pH 7.3. Lysates were centrifuged and the acid-precipitable radioactivity determined⁸. They were then treated with rabbit antisera containing antibodies against chicken Ig (RACIg). Complexes were precipitated by the addition of goat anti-rabbit Ig (GARIG). Precipitates were removed by centrifugation and the supernatants treated with anti-B serum or normal chicken serum (control) and RACIg. The anti-B serum was produced by the immunisation of B²B² chickens with B²B² cells and was extensively absorbed by red blood cells to remove non-B activity. Precipitates were washed, dissolved, and electrophoresed on 7.5% SDS-polyacrylamide gels (SDS-PAGE) at 4 mA for 16 h. Gel peaks were extracted, lyophilised, and subjected to automated sequencing on a Beckman 890C sequencer using a DMAA programme⁸. Samples from the sequencer were counted in Beckman Cocktail D. There was no interconversion of the radiolabels, as judged by back hydrolysis and amino acid analysis of portions of the immunoprecipitates⁸.

In approximately 20 separate labellings, the radioactivity in the anti-B precipitate represented 1–5% of the acid precipitable radioactivity. As shown in Fig. 1, analysis of the anti-B precipitates indicated the presence of molecules of 44,000, 26,000, and 12,000, consistent with previous studies of the chicken B antigens by Ziegler and Pink⁹. The 44,000 molecule presumably represents the H chain of the B antigen and the 12,000 molecule, the β_2 M analogue. The 26,000 MW peak, which was generally small and occasionally absent may represent the Ia analogue⁹.

As shown in Fig. 2, eight assignments were made in the N-terminal 27 residues of the molecule. Although more than one amino acid was not found at any one position, the phenylalanine at 8 and the tyrosine at 22 remain tentative, because of the significantly lower yields at these positions compared with the others. It is possible that two distinct molecules are being sequenced (analogous to the D and K ends of H-2), only one of which has a phenylalanine at position 8 or a tyrosine at position 22.

Figure 2 also compares the partial chicken B sequence with the guinea pig GPLA¹⁰, two mouse H-2 molecules^{8,11–14}, and two human HLA molecules^{15–17}. Of the five residues which are identical in the first 27 residues of H-2, HLA, and GPLA, three (residues 3, 6, 7) and possibly four (residue 8) are also identical in the chicken B

sequence. Tyr at position 27 is homologous with both mouse alleles and GPLA. The only definite difference is in the Val–Met interchange at position 12. Proline, at position 15, has not been tested in the chicken. Tyr, a tentative assignment in position 22, is homologous with H-2K^b and Arg, in position 9, shows no homology with the other proteins. This is not surprising as position 9 is presently the most variable position in transplantation antigens. The high degree of homology among those residues shared with the human, guinea pig, and mouse histocompatibility antigens strongly supports the notion that the B system, which diverged from the H-2-HLA precursor 75–200 Myr ago, is the evolutionary homologue of H-2, HLA, and GPLA.

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E. S. VITETTA
J. W. UHR
J. KLEIN
F. PAZDERKA
E. J. MOTICKA
R. F. RUTH
J. D. CAPRA

Departments of Microbiology and Cell Biology,
University of Texas Southwestern Medical School,
Dallas, Texas 75235
University of Alberta Hospital,
Edmonton, Alberta, Canada

Received 15 August; accepted 17 October 1977.

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Depurination decreases fidelity of DNA synthesis *in vitro*

DEPURINATION of DNA results from the breakage of the glycosidic bond between the purine base and the deoxyribose moiety of the purine nucleotides without disrupting the structural integrity of the phosphodiester backbone. Measurements of the rates of depurination of both synthetic polynucleotides and natural DNA in various conditions *in vitro* suggest that this damage to DNA is a frequent cellular event¹. The *in vivo* rate constant has been estimated¹ to be of the order of $1.8 \times 10^{-9} \text{ min}^{-1}$. Alkylation^{2,3} and specific glycosidases^{4,5} increase the depurination rate constants of those altered bases to $0.08\text{--}1.4 \times 10^{-3}$ and 4.0×10^{-3} , respectively. We have therefore been concerned with the possible consequences which may result during replication, repair or transcription when the template contains unrepaired apurinic sites. The biological effects of depurination are not clear. Depurination of DNA has been equated with strand breakage, a potentially lethal lesion^{6,7}, but other evidence indicates that the apurinic site may be quite stable in cells with a half life of up to several months^{8,9}. It is possible, therefore, that depurination of DNA can be a mutagenic event. Mutagenesis by depurination might occur either by an insertion of an incorrect nucleotide opposite the apurinic site or by a deletion at that point during DNA replication or repair. In view of the potentially large number of apurinic sites and their apparent stability, we investigated the effects of depurination of DNA on the fidelity of DNA synthesis *in vitro*. Recent reports suggest that modification of polynucleotide or DNA templates by various agents, including chemical carcinogens and ultraviolet irradiation, may affect the fidelity of DNA synthesis¹⁰⁻¹². We report here that depurination of the synthetic polynucleotide poly d(A-T) results in a decrease in the fidelity of DNA synthesis *in vitro* using the DNA polymerase from avian myeloblastosis virus (AMV). This is the first demonstration of a possible relationship between depurination and mutagenesis through incorporation of non-complementary nucleotides during DNA synthesis *in vitro*.

The effect of depurination of poly d(A-T) on the fidelity of DNA synthesis with AMV DNA polymerase is shown in Table 1. Poly d(A-T) was depurinated by exposure to heat and acid, and the fidelity of DNA synthesis was measured by the simultaneous incorporation of the complementary ($\alpha\text{-}^{32}\text{P}$ -dTTP) and non-complementary (^3H -dGTP) nucleotides. With the non-depurinated template, AMV DNA polymerase incorporated one molecule of non-complementary nucleotide for every 4,060 molecules of complementary nucleotide. The number of non-complementary nucleotides incorporated relative to the number of complementary nucleotides incorporated (error rate) increased in direct proportion to the extent of depurination. The error rate increased from 1/4,060 to 1/1,500 as a result of depurination (Table 1, experiment 1).

The reaction requirements for the incorporation of non-complementary nucleotide with either depurinated or non-depurinated templates are shown in Table 2. The incorporation of non-complementary nucleotide was dependent on Mg^{2+} , dTTP, enzyme and template. Elimination of any of these components markedly reduced incorporation of non-complementary nucleotide. The requirements for incorporation of non-complementary nucleotide are similar for both the depurinated and non-depurinated templates, and suggest that polymerisation is required for the incorporation of non-complementary nucleotides.

The effect of depurination on the rates of incorporation of the complementary and non-complementary nucleotides is shown in Fig. 1. The rate of incorporation of both

the complementary and non-complementary nucleotides were lower with the depurinated templates as compared with the non-depurinated template. Moreover, the rates were decreased in proportion to the extent of depurination. The coordinate incorporation of complementary and non-complementary nucleotides for each template suggests that these errors are evenly distributed during synthesis. Also, with each template, the incorporation of non-complementary nucleotide relative to the complementary nucleotide was constant with time of incubation, so the increase in error rate due to depurination was not simply the result of diminished synthesis. On the contrary, when the time of incubation was varied so that net synthesis with each modified template was the same (Table 1, experiment 2), depurination caused an absolute increase in the incorporation of non-complementary nucleotides.

The results presented here demonstrate a possible relationship between depurination of DNA and error in DNA replication. The fidelity of DNA synthesis with AMV DNA polymerase is decreased as a result of depurination of the poly d(A-T) template (Table 1, experiment 1). This misincorporation is also shown to be directly proportional to the extent of depurination (Table 1, experiment 2). Deletion experiment (Table 2) and kinetic studies (Fig. 1), suggest that the incorporation of non-complementary nucleotides is dependent on polymerisation and occurs randomly. Preliminary results indicate that a similar increase in misincorporation occurs using other depurinated templates and other DNA polymerases.

An analysis of the net mis-incorporation relative to the number of apurinic sites yields a ratio of 1 error for every

Table 1 Fidelity of DNA synthesis with poly d(A-T) template and AMV DNA polymerase

Depurination* (%)	dTMP (pmol)	dGMP (pmol)	Error rate†
Expt 1‡			
0.0	187	0.0461	1/4,060
0.6	181	0.0464	1/3,900
1.1	152	0.0419	1/3,630
1.7	130	0.0371	1/3,500
2.2	124	0.0369	1/3,360
3.4	117	0.0370	1/3,160
5.0	109	0.0375	1/2,910
6.7	94	0.0353	1/2,660
10.1	61	0.0274	1/2,220
13.4	43	0.0218	1/1,970
20.2	15	0.0100	1/1,500
Expt 2§			
0.0	100	0.0230	1/4,350
1.1	98	0.0252	1/3,890
2.2	97	0.0272	1/3,570
5.0	96	0.0323	1/2,970
10.1	102	0.0441	1/2,310

*Depurination of ^3H -dA-labelled and unlabelled poly d(A-T) templates at a concentration of 1 mg ml^{-1} was carried out at pH of 2.75 and a temperature of 55°C . The release of ^3H -adenine was measured by thin layer chromatography with cellulose plates and solvent containing *n*-butanol, isobutyric acid, 25% ammonia and water (3.0 : 1.5 : 0.1 : 1.0). The rate of depurination was 0.1% per min. No measurable depurination or change in error rate was achieved with either heat or acid treatment alone.

†Error rate = pmol dGMP incorporated / pmol dTMP incorporated.

‡Fidelity assays were performed in duplicate, were incubated at 37°C for 60 min and contained (final volume $25 \mu\text{l}$): 50 mM Tris-HCl, pH 7.8; 5 mM MgCl_2 ; 25 μM dATP; 25 μM $\alpha\text{-}^{32}\text{P}$ -dTTP (20 d.p.m. pmol^{-1}); 25 μM ^3H -dGTP (50,000 d.p.m. pmol^{-1}); 2 μg poly d(A-T) (modified as noted in the table); and 25 μg homogeneous AMV DNA polymerase. The reactions were terminated, washed and processed for determination of acid precipitable radioactivity as previously described¹³. Incorporation in the absence of incubation was 0.5 pmol dTMP and 0.0134 pmol dGMP which were subtracted from the values listed.

§Assay conditions were as described above with the exception of time of incubation which was 30 min, 40 min, 50 min, 60 min, and 120 min for the 0.0%, 1.1%, 2.2%, 5.0% and 10.1% depurinated templates, respectively.

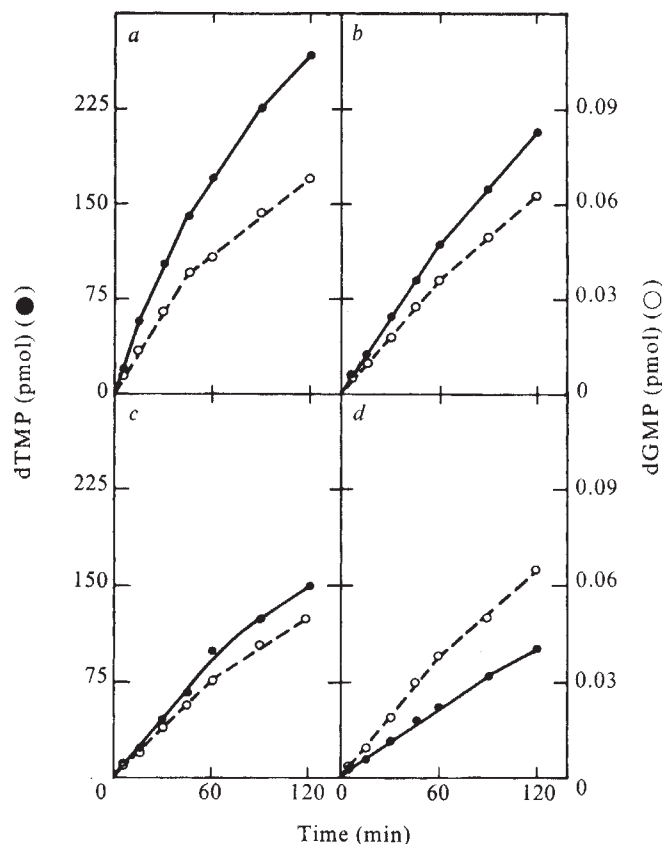


Fig. 1 Assay conditions were as described in the legend to Table 1 with the exception of time of incubation which was varied as shown. a, 0% depurination; b, 1.7% depurination; c, 5.0% depurination; d, 10.1% depurination. ●, Incorporation of dTMP; ○, dGMP incorporated.

500 apurinic sites. This frequency is lower than would occur if any one of the three nucleotides in the reaction mixture was incorporated randomly at each apurinic site. Several plausible explanations can account for this observation. First, there may be something unique about 1 in every 500 apurinic sites leading to misincorporation. Second, there may be a looping-out phenomena at most apurinic sites, producing deletions which we cannot monitor. Finally, factors other than hydrogen bonding between base pairs may be involved in base selection so as to select the complementary base rather than the non-complementary base. A detailed analysis of each of these and other possibilities is currently under investigation.

The increased mis-incorporation by DNA polymerases with depurinated templates *in vitro* raises the possibility that similar events may take place *in vivo* with mutagenic effects. A spontaneous depurination rate constant of

$1.8 \times 10^{-9} \text{ min}^{-1}$ although quantitatively small may be significant. If one assumes that the DNA of a mammalian cell contains 10^9 purine residues, this rate would produce two apurinic sites per min per cell. Alkylation in conjunction with specific glycosidases could increase this rate to more than 50 apurinic sites per min per cell. The large number of apurinic sites with miscoding potential produced by the action of chemical carcinogens, either spontaneously or in conjunction with glycosidases, may be a common mechanism of action of chemical carcinogens leading to mutations and/or carcinogenesis.

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CLYDE W. SHEARMAN
LAWRENCE A. LOEB

The Institute for Cancer Research,
Fox Chase Cancer Center,
Philadelphia, Pennsylvania 19111

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Membrane-bound carotenoid in *Micrococcus luteus* protects naphthoquinone from photodynamic action

ALTHOUGH carotenoid pigments are widely distributed in nature, their function is little understood. Studies of non-photosynthetic bacteria have led to the conclusion that these pigments are present in the plasma membrane where they protect membrane components from photo-oxidation. We report here a study of detergent-extracted carotenoid which is not protein-bound but associated with membrane lipid. The lipid-carotenoid micelles are shown to protect naphthoquinones from photodestruction, a function attributed to the native pigment.

Two major biological roles have been assigned to carotenoids in plants and prokaryotes. In photosynthetic organisms, these pigments are involved in trapping light energy¹. A more general role applicable to both photosynthetic and non-photosynthetic cells, is protection from photodynamic action. Thus in photosynthetic bacteria, carotenoid-less cells, obtained by mutation or by the use of inhibitors, were killed when illuminated in aerobic but not anaerobic conditions^{2,3}. In contrast, carotenoid-containing cells remained viable. Evidence suggested that bacteriochlorophyll acted as photosensitizer⁴. In non-photosynthetic bacteria early experiments showed that carotenoid-less cells were killed by light only when low concentrations of a suitable sensitising dye were added⁵. Subsequently, high light intensities in the absence of added sensitiser were found to kill carotenoid-less strains⁶ while carotenoid-containing cells were afforded protection. It may be postulated that a membrane-bound pigment such as a flavoprotein or cytochrome was acting as the endogenous photosensitizer⁷. In *Micrococcus luteus* (*Sarcina lutea*) a study of the effects

Table 2 Incorporation of complementary and non-complementary deoxynucleotides in various conditions with poly d(A-T) template and AMV DNA polymerase

Conditions	No depurination			13.4% Depurination		
	dTMP (pmol)	dGMP (pmol)	Error rate	dTMP (pmol)	dGMP (pmol)	Error rate
Control	113	0.0243	1/4,650	36	0.0209	1/1,720
-Mg ²⁺	0	0.0000	—	0	0.0000	—
-Poly d(A-T)	0	0.0034	—	0	0.0034	—
-dTTP	0	0.0060	—	0	0.0062	—
-Polymerase	0	0.0000	—	0	0.0000	—

Assay conditions were as described in the legend to Table 1 with the exceptions noted.

Table 1 Electrophoresis of the carotenoid extract on a sucrose discontinuous gradient

Fraction no.	Sucrose (g per 100 ml)	Carotenoid ($\mu\text{g ml}^{-1}$)	Phospholipid (mg ml^{-1})	Protein (mg ml^{-1})	Protein (g) per mole of carotenoid ($\times 10^3$)
I	21	3.5	0.46	0.02	3.4
II	21	3.4	0.41	0.03	5.3
III	21-25	1.1	0.38	0.01	5.5
IV	25	1.0	0.38	0.01	6.0
V	25	1.0	0.31	0.24	144
VI	50	0.6	0.31	0.5	500
VII	50	0.2	0.22	0.18	540
VIII	50-75	0.1	0.20	0.17	1,020
IX	75	0	0.06	0.17	—

A 15 ml sample of dialysed emulphogene extract was added to the discontinuous sucrose gradient column prepared according to the method of Hjerten¹⁹ but containing an additional sucrose layer of 21% sucrose 0.56 M NaCl. Tris-citrate buffer, pH 8.6, 0.04 M was used as buffer. 240V (13 mA) were applied to the column for 20 h at 4 °C. Fractions were withdrawn from the column as shown. Nitrogen was determined by the ninhydrin method of Jacobs²³ and the protein content calculated assuming a 16% nitrogen content and no nitrogen contribution from phospholipid. Phospholipid was derived from the phosphorus content²⁴ assuming that the phospholipid contained 4% phosphorus. Carotenoid was obtained assuming an $A_{1\text{cm}}^{1\%} = 2,500$ at 447 nm and an arbitrary molecular weight of 600.

of light on respiration in the presence of the dye toluidine blue showed that endogenous carotenoid partially protected several sites in the respiratory chain⁴. Using high light intensities and relying on endogenous sensitizers, carotenoid protected one photosensitive site, the respiratory quinone, menaquinone^{9,10}.

Although several authors have suggested that the carotenoid pigment located in the plasma membrane is specifically associated with protein or lipoprotein, this has rarely been shown^{11,12}. We have therefore attempted to isolate and purify the carotenoid pigments of *M. luteus* in a form similar to that *in vivo* using a mild non-ionic detergent, emulphogene (Mulgofen BC720). Although non-ionic detergents have been shown to remove lipid from membrane lipoproteins¹³, complete removal is not always achieved even at high concentrations^{14,15}.

Overnight shake cultures grown at 28 °C on nutrient broth were used to prepare plasma membranes by mild osmotic shock after lysozyme treatment¹⁰. The membranes were extracted with 0.6% emulphogene for 35 min at room temperature which solubilised the bulk of the major carotenoids (sarcinaxanthin, sarcinaxanthin mono-D-glucoside and decaprenoxanthin^{16,17}). The membranes themselves retained their integrity after extraction as viewed by either phase contrast or electron microscopy using negative staining (compare erythrocyte ghosts¹⁸). The extract showed a large number (at least 20) of protein bands when subjected to electrophoresis at pH 8.6 in 7.5% acrylamide gels containing 0.1% (w/v) emulphogene. The absorption maxima of the extracted carotenoid (418, 447 and 476 nm) are the same as those of the membrane-bound pigment obtained by difference spectroscopy of membranes from the wild-type and a carotenoid-less mutant. Sucrose gradient electrophoresis¹⁹ of the extract at pH 8.6 for 20 h gave fractions with carotenoid, protein and phospholipid contents shown in Table 1. The carotenoid band was diffuse, low in protein

content but rich in phospholipid. On analytical acrylamide electrophoresis at pH 8.6, the carotenoid fraction (I and II, Table 1) showed a single amidoschwartz-staining protein component running independently of the carotenoid band. In electrophoretic studies, the carotenoid band seemed translucent, was anodic between pH 3.1 and 8.6 and was evidently associated with phospholipid.

Fractionation of the extract on DEAE-cellulose at pH 8.6 resulted in a discrete carotenoid fraction running substantially in front of the flavoproteins (NADH and malate dehydrogenases). The carotenoid fraction had a substantial phospholipid content (12.5 μg per μg carotenoid) but was low in protein (2.5 μg per μg carotenoid). Partition of the emulphogene extract in a polymer system, dextran-Ficoll-polyethyleneglycol-KCNS²⁰ resulted in the carotenoid partitioning, together with the phospholipid, in the hydrophobic polyethyleneglycol phase. This phase contained protein which migrated as a single band independently of the carotenoid on acrylamide gel electrophoresis.

Using the emulphogene extract or the carotenoid phase after polymer partition and dialysis, it has been possible to demonstrate the protection of naphthoquinone (phyloquinone, vitamin K₁) by carotenoid. The quinone was added to the carotenoid preparation, briefly sonicated and assayed before and after irradiation with blue (460 $\pm$ 5 nm) light. The loss of quinone was compared with that in a similar preparation from a carotenoid-less mutant of *M. luteus* (Table 2). The loss of quinone was clearly greater in the carotenoid-less preparation. Some loss of quinone in the carotenoid system is to be expected since the method of preparation did not ensure that all quinone molecules were in association with carotenoid. By contrast, addition of quinone to an ethanolic extract of the organism did not result in protection of quinone by carotenoid.

Since in our analytical studies we find carotenoid migrating with phospholipid, it is probable that in the detergent

Table 2 Irradiation of phyloquinone (vitamin K₁) by blue light

	Before irradiation ($\mu\text{g ml}^{-1}$)	After irradiation ($\mu\text{g ml}^{-1}$)	% Loss of quinone
Quinone in extract from pigmented membranes	17.5	16.9	3
Quinone in extract from carotenoid-less membranes	17.6	13.6	23

Phylloquinone in 0.1 ml ethanol was added to a carotenoid extract after partition²⁰ into a polyethyleneglycol phase. The mixture was sonicated to form a micellar system of carotenoid and quinone. The preparation was irradiated with blue light (460 $\pm$ 5 nm) for 12 min at 15 W m⁻², the preparation having a 1 cm light path. The quinone was assayed by the borohydride reduction method of Kroger and Dadak²⁵.

extract the carotenoid is present in phospholipid micelles. The water-insoluble naphthoquinone is likely to be held in the micelles in relatively close proximity to the carotenoid, a situation not achieved in ethanolic solution. The protection of quinone by transfer of excitation energy to carotenoid will therefore be facilitated.

In studies of respiration, the function attributed to carotenoid is protection of the quinone¹⁰. Since the extracted carotenoid carries out the same function and has the same absorption maxima as in the membrane-bound state, we believe that the bulk of the pigment is associated with membrane lipid *in vivo*. In viability studies the protective function of carotenoid has been shown to depend not only on chromophore length²¹ but also on concentration²². Thus the bulk carotenoid seems to be necessary for photoprotection. This does not, however, rule out the possibility of small amounts of carotenoid being protein-bound²³ or of the pigment being associated with lipoprotein, but such states would not be essential for the protective function. We conclude that the lipid-associated carotenoid in *M. luteus* has a role in the photoprotection of naphthoquinone.

M. ANWAR
T. HASAN KHAN
J. PREBBLE
P. F. ZAGALSKY

Department of Biochemistry,
Bedford College,
London NW1, UK

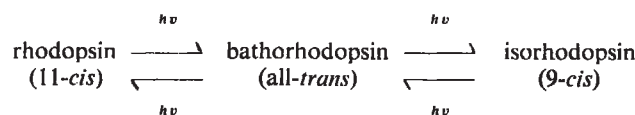
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Temperature and wavelength effects on the photochemistry of rhodopsin, isorhodopsin, bacteriorhodopsin and their photoproducts

THE primary photochemical event in visual pigments has become a matter of considerable controversy^{1–3}. We recently reviewed the various models that have been proposed and argued that the accumulated evidence strongly favours the original suggestion of Kropf and Hubbard that the primary step involves a *cis-trans* isomerisation⁴. Evidence for isomerisation is based on the photoequilibrium that can be established, both at 77 K (ref. 5) and at room temperature⁶, between rhodopsin, bathorhodopsin (its primary photoproduct), and

isorhodopsin (which contains a 9-*cis* chromophore). The equilibrium can be represented by

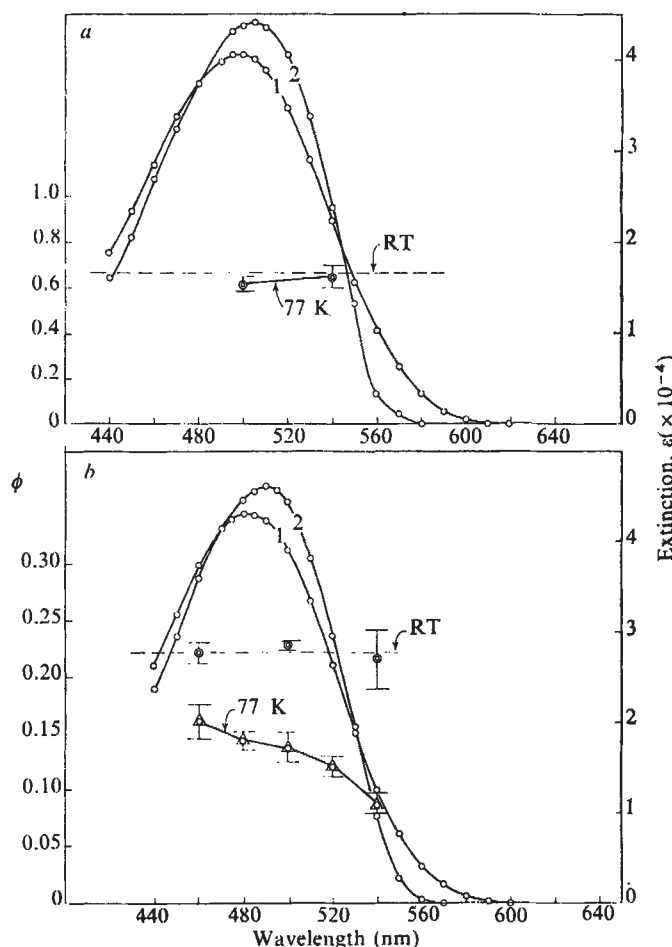


This strongly suggests that the isomeric configuration of the chromophore of the common bathorhodopsin intermediate is *all-trans* retinal, and thus light has isomerised the chromophore from 11-*cis* to *all-trans*.

It is of considerable interest to obtain a quantitative description of the physical processes involved in the primary photochemical event. Based on an analysis of the temperature and wavelength effects on the photochemistry of rhodopsin and protonated Schiff bases in solution, we proposed a model⁴ describing a potential energy curve for the excited state of the visual pigment. The major conclusion of the model is that the protein moiety of the pigment efficiently channels the excitation energy of the chromophore into a single potential minimum along the 11–12 torsional coordinate.

We extend here our original experiments and analysis to the

Fig. 1a Absorption spectra of bovine rhodopsin in 67% glycerol and 2% digitonin at (1) room temperature and (2) 77 K. Corrections were made for scattering by subtracting the spectrum of the same sample bleached in the presence of hydroxylamine. Corrections were also made for a 7.7% solvent contraction at 77 K. The dashed line represents the quantum efficiency for bleaching rhodopsin at room temperature^{10,11,12}. The circles represent the average of five measurements of the quantum efficiency at 77 K as described in the text. Standard deviations are given by the error bars. **b**, Isorhodopsin spectra under same conditions as in **a**. Isorhodopsin was prepared by regeneration of opsin with 9-*cis* retinal. Circles represent room temperature efficiencies and triangles represent the quantum efficiencies at 77 K.

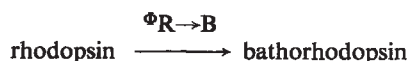


artificial pigment, isorhodopsin, and to the purple membrane protein of *Halobacterium halobium*, bacteriorhodopsin. The results for isorhodopsin provide a strong independent confirmation of the original model. Moreover, our analysis of bacteriorhodopsin photochemistry suggests a protein induced *cis-trans* isomerisation for that pigment as well.

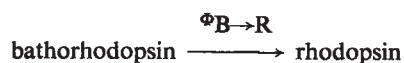
Our experimental approach is to test the effects of temperature and excitation wavelength on the absolute yield of forming the bathoproduct from both rhodopsin and isorhodopsin as well as the purple membrane protein. The amount of isorhodopsin and rhodopsin converted to bathorhodopsin by a low dose irradiation at 77 K was determined by warming the sample to room temperature (where the bathorhodopsin decays to all-*trans* retinal and opsin) and measuring the amount of unbleached pigment remaining. The amount of the purple membrane protein converted to its bathoproduct, K, was calculated from the absorption of the mixture formed after a short irradiation using the extinction coefficients of the purple membrane and K (shown in Fig. 2). Quantum yields were calculated according to Dartnall^{8,9} using rhodopsin as an actinometer and correcting for changes in extinction coefficient with temperature (Figs 1a and 1b). Quantum fluxes at longer wavelengths were determined with a rhodopsin calibrated photodiode.

As shown in Figs 1a and 2, we found that the quantum yield of the primary photo-event of rhodopsin and the purple membrane protein is, within experimental error, independent of excitation wavelength and temperature over a 220 K range. Moreover, Fig. 2 shows the quantum yield of the photoreversal from the bathoproduct K to the purple membrane pigment is also independent of temperature. On the other hand, the quantum yield for bleaching isorhodopsin (which has no wavelength dependence at room temperature) shows a significant decrease at lower temperatures (Fig. 1b). This lower quantum yield at 77 K was also wavelength dependent, the yields being smaller as wavelength increased.

The wavelength and temperature independence of the bleaching of rhodopsin strongly suggests that *cis-trans* isomerisation takes place after complete thermal relaxation and requires no activation energy. This situation may be achieved by efficiently channelling the excited system into a minimum, along a barrierless potential curve connecting the *cis* and *trans* configurations as shown in Fig. 3. This conclusion was also suggested by the observation that the quantum yields for the forward



and back



reactions nearly sum to unity⁴ which implies that most ($\phi_1 \approx \phi_2 \approx 1$) excited rhodopsin and bathorhodopsin molecules populate a common point on the excited state potential surface. (This can be easily seen since $\Phi_{R \rightarrow B} = \phi_1 \gamma_1$, $\Phi_{B \rightarrow R} = \phi_2 \gamma_2$ and $\gamma_1 + \gamma_2 = 1$. Thus, $\Phi_{R \rightarrow B} + \Phi_{B \rightarrow R} = 1$ only when $\phi_1 \approx \phi_2 \approx 1$).

The general form of the potential curve shown on the left side of Fig. 3 is amenable to more quantitative analysis. Using the absolute and relative quantum yields at 77 K (see Fig. 3) we find $\phi_1 = 1.0$, $\phi_2 = 0.9$, $\gamma_1 = 0.67$ and $\gamma_2 = 0.33$. Moreover, the temperature independence of the $R \rightarrow B$ photoisomerisation shows that these values are accurate at room temperature as well (with the possible exception of ϕ_2 which cannot be determined at room temperature). Thus, the left side of Fig. 3 provides the first detailed characterisation of the primary photochemistry of rhodopsin.

The fact that $\phi_2 = 0.9$ (which requires that $\Phi_{R \rightarrow B} + \Phi_{B \rightarrow R}$ be slightly less than unity) is due to the leakage (ϕ_3) of bathorho-

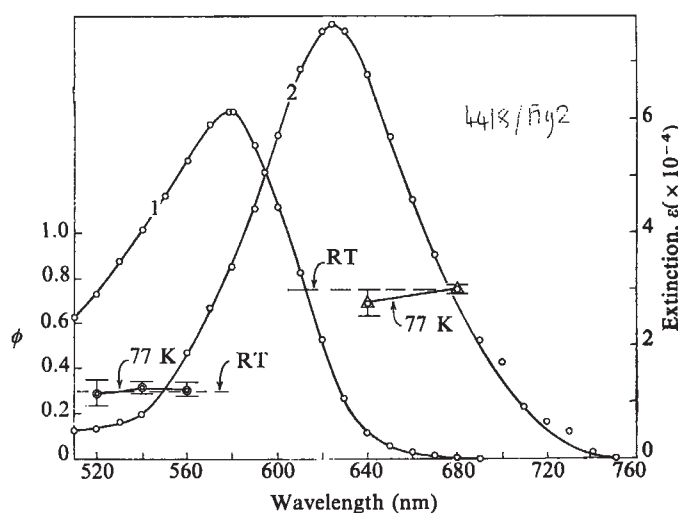


Fig. 2 Absorption spectra at 77 K in 67% glycerol of (1) the purple membrane protein and (2) its primary photoproduct, the K intermediate. The K spectrum was determined from the spectrum of the 500 nm photosteady-state mixture, assuming it to be 28% K. This was determined by warming the mixture under conditions⁹ which allow the complete conversion of K to M(412), revealing the percentage of pigment unconverted in the photosteady-state mixture. The circles represent the purple membrane protein quantum efficiency and the triangles represent the K quantum efficiency both at 77 K. The dashed lines represent the corresponding quantum efficiencies for the pigment (at -40°C (ref. 9); a similar value is seen at room temperature)¹⁴ and for the bathoproduct (at room temperature RT).

dopsin to the 9–10 torsional coordinate leading to the formation of isorhodopsin. An independent check of the numbers given above requires that an analysis of the $B \rightleftharpoons I$ interconversion yield $\phi_3 = 1 - \phi_2 \approx 0.1$. Unfortunately, the temperature dependence of $\Phi_{I \rightarrow B}$ and its wavelength dependence at 77 K (due perhaps to a small barrier along its potential surface) preclude an accurate determination of ϕ_3, ϕ_4, γ_3 and γ_4 . It is possible, however, to use the values of $\Phi_{I \rightarrow B} = \phi_4 \gamma_4$ and $\Phi_{B \rightarrow I} = \phi_3 \gamma_3$ to estimate ϕ_3 .

Taking an average value of $\Phi_{I \rightarrow B} \approx 0.13$ (Fig. 1b) and the ratio $\Phi_{B \rightarrow I} / \Phi_{I \rightarrow B} = 0.4$ determined previously⁴ we find $\phi_3 \gamma_3 = 0.05$. (Thus our model yields $\Phi_{B \rightarrow R} / \Phi_{B \rightarrow I} = \phi_2 \gamma_2 / \phi_3 \gamma_3 \approx 5$, in complete agreement with the estimate of Yoshizawa and Wald⁵.) Simple numerical considerations (see Fig. 3a) now lead to the conclusion that ϕ_2 must be approximately 0.1, thus providing an important consistence check for the analysis of the $B \rightleftharpoons R$ interconversion.

The most striking implication of the above analysis is the complete channelling ($\Phi_1 = 1.0$) into the common minimum from excited rhodopsin molecules (11-*cis* configuration) and almost complete channelling of bathorhodopsin (all-*trans*) ($\Phi_2 = 0.9$) to the 11–12 torsional coordinate accounting for the relationship $\Phi_{R \rightarrow B} + \Phi_{B \rightarrow R} \approx 1$. As can be seen from Fig. 2, the same relationship ($\Phi_{PMP \rightarrow K} + \Phi_{K \rightarrow PMP} = 0.28 + 0.72 = 1$) also characterises the primary photoevent in light-adapted bacteriorhodopsin, both at 77 K and at room temperature (Fig. 2 and refs 9, 13, 14). Moreover, the photoreversibility of the various intermediates so characteristic of the bleaching sequence of visual pigments⁵ is also a feature not only of the K intermediate but also of the 'M' (412 nm) intermediate of the purple membrane protein¹⁰. These striking photochemical analogies very strongly suggest a *cis-trans* geometry change as the primary photochemical step in bacteriorhodopsin.

The extremely simple photochemical behaviour of both rhodopsin and purple membrane protein should be contrasted with the complex patterns that characterise the photoisomerisation of retinal analogues¹⁵ and other well studied systems such

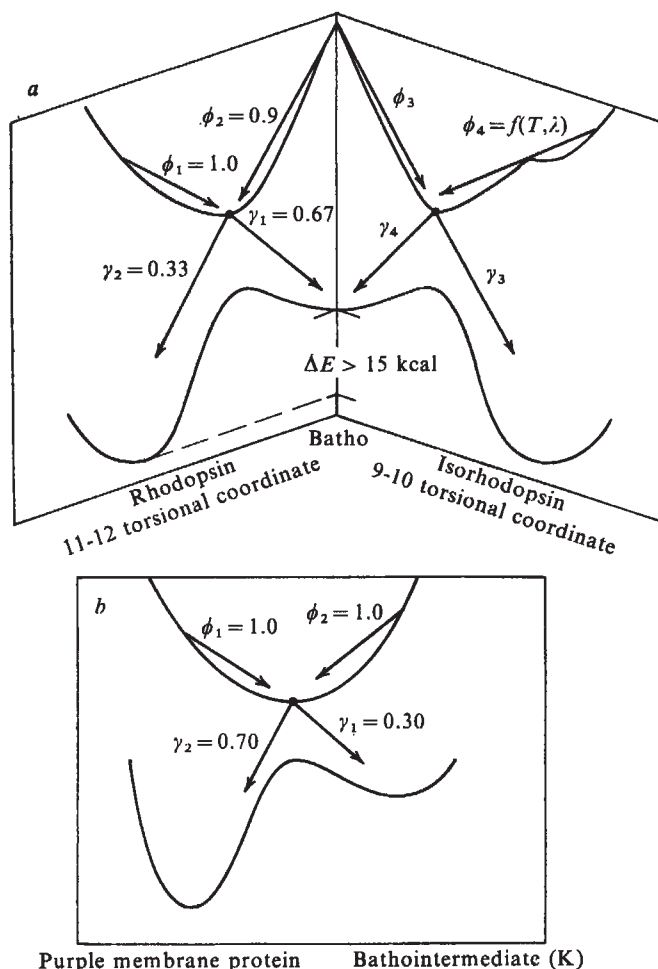


Fig. 3a Potential energy diagrams for the rhodopsin, bathorhodopsin, isorhodopsin interconversions. The quantum efficiency for rhodopsin isomerisation is $\Phi_{R \rightarrow B} = \Phi_1 \gamma_1$, where Φ_1 is the probability of reaching the bottom of the potential well of the excited state and γ_1 is the probability of going to bathorhodopsin from the bottom of the well. Similar relations hold for $\Phi_{B \rightarrow R}$, $\Phi_{B \rightarrow I}$, and $\Phi_{I \rightarrow B}$. Quantum yields for the 11-12 coordinate are calculated from the 77 K data presented here and using $\Phi_{R \rightarrow B}/\Phi_{B \rightarrow R} = 2.2$ (ref. 4). For the 9-10 coordinate, our room and low temperature data set limits for Φ_1 between 0.22 and 1.0 leading to $0.22 < \gamma_4 < 0.59$, $0.41 < \gamma_3 < 0.78$ and then using $\Phi_3 \gamma_3 = 0.06$ we find $0.07 < \Phi_3 < 0.14$. Thus, all values of Φ_3 are consistent with $\Phi_3 \approx 0.9$. b, Hypothetical energy level diagram for the purple membrane protein and its batho-product K. This diagram ignores the observed fluorescence which is thought to come from another excited state (ref. 19).

as stilbenes¹⁶. In contrast to the pigments, the quantum yields of the model compounds are strongly wavelength-dependent and can be 1-2 orders of magnitude less than those of the pigments. Thus, it seems that the protein facilitates isomerisation in the chromophore by altering the excited state potential energy surfaces and/or by modifying rates of radiationless deactivation. It is interesting in this context that the artificial pigment isorhodopsin seems to undergo a less efficient and more complex photochemistry than does rhodopsin.

The protein can also influence the ground state energy surfaces. Although free 11-*cis* and all-*trans* retinal have very similar free energies¹⁷, the opsin modifies the equilibrium conformation of the chromophore so that rhodopsin is at least 10 kcal mol⁻¹ lower in free energy than the final products, opsin and free all-*trans* retinal. Moreover, bathorhodopsin decays spontaneously to opsin + all-*trans* retinal and, thus, must be still higher in free energy than these products; this free energy must be obtained from the photon absorbed by

rhodopsin. A hypothetical ground state energy diagram is also included in Fig. 3.

The bathoprocess of the purple membrane protein must also contain a significant fraction of the absorbed photon's energy since the spontaneous decay of this photoproduct back to the original pigment is used to power a proton pump across the cell membrane¹⁸. In view of the basic similarity of this pigment and rhodopsin with respect to the chromophore structure and binding and their primary photochemistry, it is reasonable that similar energy storage mechanisms are operative in both pigments.

Recently, Peters *et al.*²⁰ have observed a deuterium-dependent decay of a transient species following picosecond stimulation of rhodopsin; they have proposed that light does not isomerise the chromophore, but rather initiates a proton transfer to it (see also ref. 3). We do not, however, believe that their data in any way rules out *cis-trans* isomerisation; indeed many interpretations of the data, other than the one they give, are possible. For example, the transient species may be due to a time-dependent shift in the absorption spectrum of bathorhodopsin, due to a relaxation of the protein/chromophore following isomerisation; that is, it could arise if the proton of the protonated Schiff base changes its orientation with respect to its counter ion or even switches its counter ion as a result of *cis-trans* isomerisation. This could account for the temperature and deuterium effects of the rate of formation of bathorhodopsin. In any case, the models Peters *et al.* propose for the primary event are incompatible with the known properties of bathorhodopsin discussed above and in ref. 4. Moreover, it is difficult to see how proton tunnelling as the only primary event would result in a stable species (at 77 K) which would not revert back to rhodopsin. Finally, Green *et al.*²¹ have recently provided evidence that isomerisation in rhodopsin and isorhodopsin can occur in picoseconds at room temperature.

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JAMES B. HURLEY
THOMAS G. EBREY

Department of Physiology and Biophysics,
524 Burrill Hall,
University of Illinois,
Urbana, Illinois 61801

BARRY HONIG
MICHAEL OTTOLENGHI

Department of Physical Chemistry,
The Hebrew University,
Jerusalem, Israel

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matters arising

Membrane initiation of DNA synthesis

HOBART *et al.*¹ reported convincing autoradiographic evidence that DNA replication is initiated near or on the nuclear membrane in sea urchins. In their discussion of the difference between their results and those reported by others for mammalian cells²⁻⁴, they raise the possibility that the latter results might arise from repair synthesis because, "... autoradiographic analysis cannot distinguish between replication and repair synthesis of DNA ..." and "Such a situation would confuse the interpretation of autoradiographs in determining sub-nuclear sites of replication".

It is only remotely possible that this explanation is correct. DNA repair synthesis in undamaged mammalian cells is not detectable by even very sensitive methods⁵, let alone autoradiography. In cells whose DNA molecules are severely damaged, repair synthesis (excision) never exceeds about 1% of the amount of semiconservative synthesis that would occur simultaneously in undamaged cells^{6,7}; even this amount of repair synthesis would not be detectable by the electron microscope autoradiographic method used by Hobart *et al.*¹.

Thus, the explanation for the results showing a difference between sites of initiation of DNA synthesis in sea urchins¹ and in mammalian cells²⁻⁴ is very likely other than the one proposed by Hobart *et al.*¹.

ROBERT B. PAINTER

JAMES E. CLEAVER

Laboratory of Radiobiology,
University of California,
San Francisco, California 94143

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HOBART *et al.* REPLY: We agree with Painter and Cleaver that it seems only remotely possible that repair synthesis

of DNA could account completely for the differences between our results with sea urchins and those of others using mammalian cells. But, it remains to be determined if repair synthesis is indeed a minor activity in cells which have been maintained for prolonged periods of time in tissue culture and have been treated with various metabolic inhibitors to achieve synchronisation. In any case the results with sea urchin embryos, which are normally synchronous and in which semiconservative replication is undoubtedly the principle activity, indicate that DNA synthesis occurs on or near the nuclear membrane.

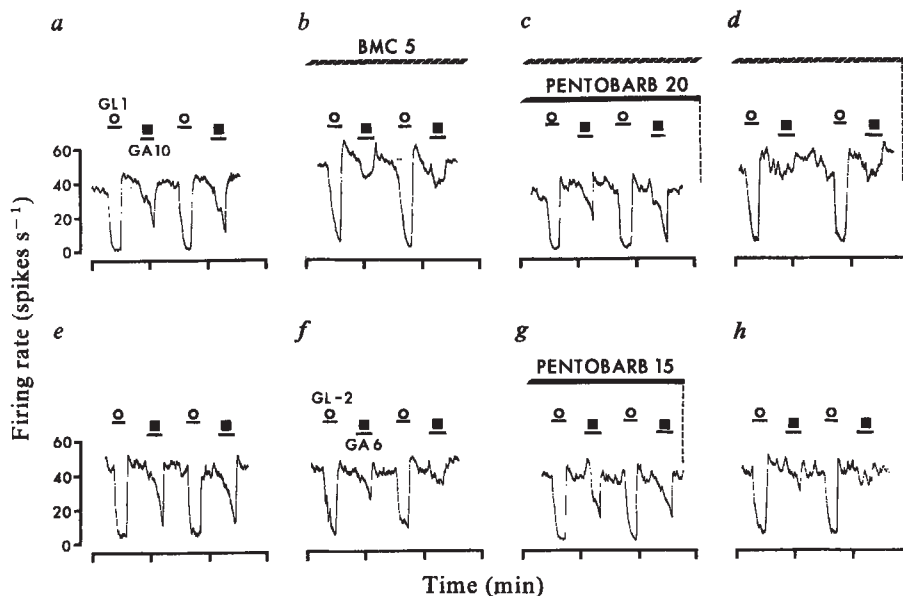
Pentobarbitone enhancement of the inhibitory action of GABA

BOWERY and Dray¹ have suggested that pentobarbitone reverses the effects of the γ -aminobutyric acid (GABA) antagonist bicuculline methochloride (BMC), on both the superfused rat

superior cervical ganglion and rat medullary neurones *in vivo*, without potentiating the action of GABA on these tissues in the absence of BMC. Such an action does not readily account for the accentuation by pentobarbitone of inhibitions mediated by GABA in the mammalian central nervous system², except by antagonism of an as yet undetected endogenous GABA antagonist.

We have investigated the action of pentobarbitone on 31 dorsal horn interneurons and three Renshaw cells of eight low spinal cats anaesthetised i.p. with either α -chloralose and urethane (40 and 400-800 mg per kg), diallyl barbituric acid and urethane (60 and 600 mg per kg) or pentobarbitone sodium (35 mg per kg). Amino acids, acetylcholine, and pentobarbitone were administered electrophoretically from solutions in the outer barrels of seven-barrel micropipettes, the 3.6 M NaCl-containing centre barrels of which were used to record extracellular action potentials of single neurones. The following solutions were

Fig. 1 Ratemeter records of the firing of a dorsal horn interneurone in a cat anaesthetised with α -chloralose and urethane. The electrophoretic ejection of glycine (GL) and GABA (GA) are indicated by the appropriate symbols, horizontal black lines and currents (nA). *b* and *c* were recorded 5 and 10 min after the commencement of the ejection of BMC which continued for 15 min, ceasing after *d*, as indicated by the broken vertical line. The ejection of pentobarbitone (PENTOBARB) commenced 3 min before *c* and again 2 min before *g*, and was also terminated at the vertical broken lines. Records *e* and *h* were 1 min after *d* and *g* respectively. Ordinates, firing rate, spikes s^{-1} , abscissae, time in min.



used: GABA (0.1, 0.2 M; pH 3), glycine (0.5 M; pH 3), L-glutamate (0.5 M; pH 7.5), DL-homocysteate (DLH, 0.2 M; pH 7.5), acetylcholine hydrobromide (ACh, 0.25 M), BMC (10 mM in 165 mM NaCl) and sodium pentobarbitone (20 mM; pH 9.5 or 250 mM in a 7:2:1 mixture by volume of water, propylene glycol and ethanol).

In confirmation of Bowery and Dray¹, pentobarbitone (10–60 nA) partially reversed the antagonism by BMC of the inhibition of cell firing by GABA. With most neurones, however, in the absence of BMC, pentobarbitone ejected with the same or even lower electrophoretic currents enhanced sub-maximal inhibition of firing by GABA. Furthermore, the inhibitory action of electrophoretic glycine on many cells was also enhanced, but not to the same extent as that of GABA.

Results from one neurone are illustrated by the records in Fig. 1 of the rate of firing of a dorsal horn interneurone maintained by the continuous ejection of DLH, 8 nA. Firing was inhibited by glycine and GABA, ejected consecutively each for 7 s at fixed time intervals (Fig. 1a). During the ejection of BMC, the effect of GABA was reduced (Fig. 1b), but was restored to near control values during the simultaneous administration of pentobarbitone from an aqueous solution (Fig. 1c). This effect of pentobarbitone was reversible (Fig. 1d), as was the antagonism of GABA by BMC (Fig. 1e). Several minutes later, when the currents ejecting glycine and GABA had been reduced, in particular that of GABA to reproduce the inhibitory action observed during the ejection of BMC (compare Figs 1b, d and f), pentobarbitone also reversibly enhanced the effectiveness of GABA (Fig. 1g and h). The degree of enhancement of the GABA effect by pentobarbitone in the absence of BMC was very similar to that during the ejection of BMC.

Effects such as these, which were observed with spontaneously active cells as well as with those in which firing was maintained with DLH, glutamate or ACh, and irrespective of the anaesthetic used or whether BMC was in one of the barrels of the micropipettes, render unnecessary a postulate that pentobarbitone displaces BMC from receptors on central neurones¹. At least in the spinal cord of the cat the increased effectiveness of GABA by pentobarbitone probably is adequate to account for the reversal of antagonism by BMC. Further experimentation will be required to determine whether this action of pentobarbitone results from interference with the cellular uptake of amino acids or from a more direct effect at postsynaptic receptors³. The importance of this latter type of action

is suggested by the frequent observation that electrophoretic pentobarbitone depressed the firing rate of neurones, and that pentobarbitone, like GABA, depolarises the terminals of group Ia afferent fibres in the cat cord (our unpublished work).

D. R. CURTIS

D. LODGE

*Department of Pharmacology,
John Curtin School
of Medical Research,
PO Box 334,
Canberra City Act 2601, Australia*

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BOWERY and DRAY reply—The results of Lodge and Curtis are most interesting but, whilst we do not dispute that enhancement of responses to GABA and glycine can occur in the absence of bicuculline methochloride (BMC), in our experiment in the rat medulla we saw no clear enhancement of sub-maximal responses to GABA or glycine using expelling currents of pentobarbitone which on the same cells reversed the antagonism produced by BMC or strychnine. Pentobarbitone in all cases did not decrease the background firing rate of the cell, an observation which differs from that of Lodge and Curtis. Only when we increased the ejecting current by 2–3-fold did pentobarbitone decrease the firing rate¹. It remains to be seen whether this decrease in firing results from a GABA-mimetic effect at post-synaptic receptors. It is interesting to note that the increase in firing rate produced by BMC in the Lodge and Curtis experiment (shown in their Fig. 1b) seemed to be antagonised by the ejection of pentobarbitone (c).

Although Lodge and Curtis suggest that the enhancement of responses to

GABA could account for the observed BMC reversal on dorsal horn interneurones in the cat spinal cord we believe that under our experimental conditions on spontaneously-active neurones in the rat medulla, these phenomena are separable. Some evidence in support of this comes from results we have obtained with other drugs in the superior cervical ganglion. Although the barbiturates were the most effective in reversing BMC antagonism other central depressant drugs with quite different chemical structures, for example, benzodiazepines, amitriptyline and promethazine also reduced BMC antagonism. None of these substances significantly potentiated the effect of GABA at the concentrations employed nor did they exhibit any direct GABA-mimetic activity even at >10-fold higher doses.

Experiments with nipecotic acid in the ganglion indicate that BMC reversal by pentobarbitone is probably unrelated to any inhibition of the cellular uptake of GABA. Nipecotic acid inhibits GABA uptake in this tissue and thus potentiates the response to GABA². Unlike pentobarbitone, however, nipecotic acid will neither prevent nor partially reverse BMC antagonism and moreover will not prevent pentobarbitone reversing the action of BMC.

Two phenomena associated with the action of pentobarbitone in relation to GABA and glycine receptors have been described—first, an enhancement of the action of GABA or glycine as reported by Lodge and Curtis and others^{3,4} and second, a direct GABA-mimetic action as described by Nicoll⁴. Our results suggest that a third phenomenon may occur, that of reversal of the action of convulsants which antagonise responses to GABA and glycine.

Although separation of these phenomena may not be easy in some systems our results indicate that it is possible in the rat medulla and sympathetic ganglion and may depend on the concentration of pentobarbitone employed.

N. G. BOWERY

*Department of Pharmacology,
St Thomas's Hospital Medical School,
London SE1, UK*

A. DRAY

*Department of Pharmacology,
The School of Pharmacy,
29/39 Brunswick Square,
London WC1, UK*

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Matters Arising

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reviews

Best kind of Natural History

S. R. J. Woodell

Population Biology of Plants. By John L. Harper. Pp. xxiv+892. (Academic: London, New York and San Francisco, 1977.) £30; \$58.60.

As Professor Harper states in his preface, population biology was for almost half a century the almost exclusive preserve of zoologists. In the early part of this century, Sukatschew, Tansley and especially Clements experimented on plant populations, but thereafter most plant ecologists turned to other aspects of the subject. This might seem strange. Plants are immobile. They do not have to be captured, marked and recaptured, but whereas animals respond to environmental changes by changing numbers, plants may change in either number or size, or both. It is perhaps this plasticity that has deterred some botanists from studying their populations. Numbers can be counted, size has to be measured, usually destructively.

Harper was fortunate to come into contact early in his career with Charles Elton and his group in Oxford, and then with agronomists in Aberystwyth, to whom experimentation on plant populations was nothing new. Unlike most botanists he realised the relevance of animal population studies to plants, and acted upon it. During the past twenty-five years he, his students and co-workers have done more than anyone to set the science of plant population biology on its feet, and it is appropriate that he should write the first major book about it.

A short introductory chapter on "Experiments, Analogies and Models" sets the scene, and is followed by five major sections: on "Dispersal, Dormancy and Recruitment"; "The Effects of Neighbours"; "The Effects of Predators"; "The Natural Dynamics of Plant Populations"; and finally, "Plants, Vegetation and Evolution".

Each is complete in itself, but they are all linked by the evolutionary theme that pervades the whole book. Harper, in acknowledging his debt to Darwin, states that he thinks the third chapter of the *Origin of Species* is the best ecological text ever written. Though not all would agree with this, there is no doubt that it shows an insight into interactions between organisms that has not yet been surpassed, for all that it was written 120 years ago. Harper comments (p492): "in ecology . . . the idea of evolution as a continually operating force in ecological interactions seems to be accepted unwillingly." Sadly, this is true of many, though not by any means all ecologists; such workers as G. E. Hutchinson are outstanding exceptions. This book puts the relationship between ecology and evolution firmly back into the centre of the stage.

Every chapter in Harper's book has something to say about how plant populations interact with their total environment. With animal ecologists in mind as readers, he is constantly pointing to ways in which animal and plant populations are similar and different. Apart from the plasticity of plants, two especially interesting points he makes are that the food of green plants does not reproduce and is not the result of a reproductive process (this fundamentally affects their population biology); and that plants consist of "populations of parts" (and this cannot be said of animals except perhaps corals, and some colonial Hymenoptera).

Everyone will find much of interest here; there are ideas enough to keep several workers busy for a lifetime. If I may pick chapters which especially interested me, I would select, chapter 11, "Mechanisms of Interaction Between Species", as a most stimulating, critical and thought-provoking discussion of the problems and pitfalls

inherent in studying plant interactions. The several chapters on "The Effects of Predators" comprise an account in depth of some aspects of plant-animal interactions, a fashionable field these days after long neglect. The final section, where evolution is brought to the fore, is a fascinating development in evolutionary terms of much that has gone before.

It will be clear that I liked this book; indeed, I found it difficult to put down once I had begun it. There are small irritations. In places, it reads rather like a catalogue of experiments, though every experiment described is very relevant. Those familiar with Harper's work will be familiar with much of the content of the book, but to have it all together is very valuable; and a glance at the bibliography reveals the breadth of the literature surveyed here. Harper is not quite consistent in his acceptance in some places, and rejection in others, of anecdotal evidence. These are minor flaws. A credit is the inclusion of good chapter summaries at the beginning of the book.

'Natural History' is a much-abused term, probably largely as a result of the flood of poor quality writings on it during the last century. Yet who would deny that *The Natural History of Selborne* (Gilbert White) is a great book, or that although much of what Darwin wrote was Natural History, he was a great scientist. The Oxford English Dictionary defines Natural History thus: "A work dealing with the properties of natural objects, plants or animals; a scientific account of any subject on similar lines." In those terms, I am sure that Professor Harper will not object to his book being described as an example of the best kind of Natural History. □

S. R. J. Woodell is Lecturer in Ecology in the Botany School at the University of Oxford, UK.

Polymer science

Macromolecules. Vol. 1: Structure and Properties. Pp. 532. Vol. 2: Synthesis and Materials. By Hans-Georg Elias. Pp. 131. (Wiley: London; Plenum: New York, 1977.) £24; \$42.50 each volume.

In his preface, the author describes these two volumes as a textbook, treating "the whole field of macromolecular science, from its chemistry and physics to its applications, in a not too elementary manner." To attempt this is a stupendous undertaking for a single author, but it can be said at once that the text amply justifies the claim. The original version (1971) was in German and the present text is a translation from a 1975 revised German edition—but shows no internal evidence of not having been written in English from the start.

The method adopted is that of straightforward exposition; where differing viewpoints might be held, the author usually takes a clear line, no doubt with the aim of avoiding confusion for the student. The area to be covered is vast, and within the scope of 1,100 pages most topics can receive no more than outline treatment. To enable the student to supplement this, a reading list of books and review articles is provided for each section, but there are no references to the original literature (except for historical introduction). The opportunity has evidently been taken in revision and again in translation to add recent titles to the original reading lists.

The contents of the two volumes can be described roughly as physics and chemistry, respectively; and although the work is planned as a whole, the two parts could each stand alone. Volume 1 deals with the theoretical and experimental basis of our knowledge of polymer structure, and physical properties. It is divided into three parts: structure, solution properties, and solid-state properties. Little is assumed in the way of background knowledge: chapter 5 opens with an account of X-ray scattering; and chapter 9.5 with a presentation of the basic principles of light scattering. Experimental methods are outlined, and although equipment is not described in detail, key features are frequently pointed out. Although this volume is largely concerned with principles, it is sufficiently wide-ranging to include reference to such practical matters as adhesives and glues, and the testing of insulators for resistance to tracking.

Volume 2 divides into two roughly equal parts dealing, respectively, with principles of polymerisation and individual polymers. Following an introductory chapter on general principles, the first part deals successively with polycondensations; ionic, insertion and radical

reactions, copolymerisation; and finally with reactions of macromolecules (including aging). This is a wide-sweeping survey at a very well chosen level.

The last section of the book is entitled "Polymers" and is divided into nine chapters, of which the first four deal broadly with synthetic organic polymers classified according to their main chains: carbon, carbon-oxygen, carbon-sulphur, carbon-nitrogen. The chapters are further broken down by polymer type into 25 sections, many of them further subdivided. The general method of treatment for each polymer includes monomer synthesis, polymerisation methods and mechanisms, polymer properties and reactions; all done very competently and succinctly. Final chapters extend the scope to biopolymers and inorganic chains, and serve at least to remind the student of the wide ramifications of macromolecules in nature.

Nothing could be easier for the reviewer of a work of this kind than to select a field in which he is personally expert and

to point out shortcomings and possible errors of judgement. Inevitably, when an author undertakes to review such a vast field he will succeed better with some areas than others. What is more pertinent to ask is (a) whether the work succeeds in its declared aim, and (b) what other needs it might serve. On the first of these counts, there is no doubt that any student who works systematically through this text will acquire a broad knowledge and understanding of polymer science and its applications. Spread over the three years of a Ph.D. course, it would provide a superb accompaniment to the experimental study of a limited topic. For the reviewer, its role will be that of an admirable first source of information, providing a coherently argued introduction and a guide to further reading. On both counts, it is a notable addition to polymer literature.

Geoffrey Gee

Geoffrey Gee is Professor of Chemistry at the University of Manchester, UK.

Volcanic leitmotive

Carbonatite-Nephelinite Volcanism. By Michael J. Le Bas. Pp. 347. (Wiley: London, New York and Toronto, 1977.) £22; \$44.

THERE is a long standing joke that one can doze off during one of Wagner's *Ring of the Nibelungen* operas, only to wake up ten minutes later to find the same singer standing on the same spot singing the same song. One gets the same impression from Le Bas' book. No matter where one dips into it, there is a certain sameness about the text. Tortuous rock names like uncomphagrite, turjaite, melteigite, ijolite and urtite keep cropping up in the prose like Wagnerian leitmotive. To appreciate the *Ring*, one has to be able to recognise the leitmotive. The same is true of this book: without a firm grasp of the convoluted terminology of hyper-alkalic volcanism, one is lost.

The first sentence of the first paragraph of the text starts: "The classic igneous intrusions of Fen (Brogger, 1921; Sæther, 1957) and Alno (Eckermann, 1948) in Scandinavia, of Magnet Cove, Arkansas (Erickson and Blade, 1963), of Iron Hill, Powderhorn, Colorado . . .". This bleak style is relentlessly preserved throughout the 24 chapters of the book. Nineteen of these are detailed descriptions of individual areas or volcanoes in East Africa written by Le Bas and his colleagues who worked in the area

from 1963 to 1969. Many of these chapters are based on the distilled and condensed Ph.D. theses of research students.

Of most interest to this reviewer was the third chapter, in which Le Bas unravels the nomenclature of the rock suites—this is essential reading and should come before chapter 1—and the last two, which cover magmatic and metasomatic processes and the petrogenesis of the carbonatite nephelinite suite. There are two appendices, containing geochronological and major element geochemical data; there are few trace element data.

Le Bas has produced a thoroughly scholarly work summarising many years of work by him and his colleagues in East Africa, and one which is unequivocally aimed at a specialised readership. The descriptive chapters will undoubtedly provide a valuable source of information to workers studying alkalic volcanism in East Africa and it is clearly useful to have all this data published under one cover. It is a pity, however, that Le Bas did not broaden his scope slightly. There must be many potential readers, not directly involved in the field, who would be glad of an up-to-date and intelligible review of the fascinating petrological problems posed by the carbonatite-nephelinite suites. Only a small proportion of this book is likely to interest them.

Peter Francis

Peter Francis is Lecturer in the Department of Earth Sciences at the Open University, Milton Keynes, UK.

Molecular trees

Molecular Anthropology: Genes and Proteins in the Evolutionary Ascent of the Primates. Edited by M. Goodman, R. E. Tashian and J. H. Tashian. Pp. 466 (Plenum: New York and London, 1977.) \$42.

THIS volume contains 20 chapters, most of which were presented at a symposium in Austria during July 1975. The chapters are grouped under five headings: "Background to Some Key Issues"; "Molecular Evolution as Interpreted by Mathematical Models"; "Primate Phylogeny and the Molecular Clock Controversy"; "Primate Evolution Inferred from Amino Acid Sequence Data"; and "Multigene Families and Genetic Regulation in the Evolution of Man". Forty-nine authors contributed to the compilation, which provides an excellent interplay between scientists in various fields, especially biochemistry, anthropology and genetics.

There is a chapter by E. L. Simons, a palaeontologist, on the fossil record of primate phylogeny, and one on splitting times among hominoids as deduced from the fossil record, by Alan Walker. These provide an interesting and necessary counterpoint for a host of molecular and biochemical phylogenetic trees that are constructed, by authors of other chapters, on the basis of differences between homologous proteins. Various monkeys and other vertebrates have kindly furnished the proteins, especially globins, whose amino acid sequences and immunological cross-relationships are used for the construction of these trees. There is also a short chapter on satellite DNA and ribosomal genes in primates. G. W. Lasker gives us his ideas on what "molecular anthropology" is, with the help of an imaginary dialogue.

The use of comparisons of protein sequences for measuring evolutionary divergence in primates, to which most of this book is devoted, depends on acceptance of the concept of the "molecular evolutionary clock". This concept states that amino acid substitutions in protein molecules take place at an approximately uniform rate during evolution. There is much debate as to how "uniform" this rate is for a given protein. The general consensus is that it runs at different rates at various times.

Most molecular evolutionists use the "clock" to some extent because of its obvious relationship to phylogeny.

In chapter 2, Vogel, Kopun and Rathenberg discuss transitions and transversions in haemoglobin variants. They say that: "CT transitions in the DNA code corresponding to AG transitions in the mRNA code are more frequently observed than would be expected with random substitutions." So-called "CT transitions in the DNA code" are, however, actually base-pair substitutions in which there has been an interchange between an A.T pair and a G.C pair. The decision as to which DNA strand is transcribed depends on which strand contains the binding site for RNA polymerase; and this decision is unaffected by the amino acid composition of the protein that is subsequently synthesised. There is no way of deducing, from an amino acid substitution, which member of the corresponding DNA base-pair underwent the mutation, and which was changed when replication next took place. As the authors correctly note, their compilations are indeed biased, because haemoglobin variants are detected

by electrophoresis, which does not measure changes that are not accompanied by an alteration in charge. In their Table 9, Vogel *et al.* have written alanine codons as the complementary DNA codons. To be consistent with polarity of DNA strands, they should have been written from right to left. Also, one of the codons is wrong.

In Chapter 11, by Matsuda, Table 4 shows the amino acid exchanges and minimum mutation distances in the opposite order from the caption to the table. Chapter 17, by Goodman, presents a lucid account of primate genealogy as deduced from globins and cytochrome *c*.

The book contains extensive compilations of amino acid substitutions in homologous proteins, especially globins, obtained from various primates. It also contains chapters on random and non-random processes in molecular evolution, maximum parsimony, carbonic anhydrase, antibody specificity and gene action.

Thomas H. Jukes

Thomas H. Jukes is Professor of Medical Physics at the University of California at Berkeley.

Expertise in analytical chemistry

Analytical Chemistry: Essays in Memory of Anders Ringbom. Edited by E. Wannien. Pp. xiv+607. (Pergamon, Oxford and New York, 1977.) £27.50.

THIS volume consists of a judicious mixture of authoritative reviews, stimulating and often entertaining essays, and a sprinkling of original papers. The topics are spread across the face of modern analytical chemistry with particular stress on those areas with which the late Professor Anders Ringbom has been most actively associated.

The most homogeneous section is the first, containing some sixteen articles on or related to the stability constants and structures of complexes in solution. They succeed in presenting a very adequate and well-blended picture of the present state of the art, with a significant proportion devoted to the question of mixed complexes which have been so actively investigated in the last decade. In the various reviews, the many technical problems which can so readily disturb or mislead the newcomer to this rather exacting field of study are laid out clearly and amply documented. This section should thus be of particular value to the non-specialist who wishes to update himself, but will I am sure also be read with considerable interest by those with long experience and greater expertise.

The second section is composed of eight articles on various titration procedures and three on colorimetric indicators. This is followed by four articles on aspects of electrochemistry (polarography, electrometric titrations and ion-selective electrodes), five on separation techniques (metal chelates in gas-liquid chromatography, ion-exchangers and extraction), seven on trace analysis (for example, activation, anodic-stripping voltammetry, fluorescence and atomic absorption) and three on kinetic methods of analysis. Finally, there is a miscellaneous section with articles on sampling, the writing of a scientific paper, photoelectron spectroscopy, statistical analysis, capillary electrophoresis, and the history of analytical chemistry.

The 600 closely packed pages provide a curious and varied mine of information and expertise in analytical chemistry, and the list of authors (for example, Kolthoff, Bates, Reilly, Freiser, Margerum, Elving, Laitinen, Anderegg, Beck, Nasanen, Osterberg, Perrin, Pribil, Bjerrum, Irving, Tanaka, Pungor, Alimarin, Yatsimirskii, and Flaschka) contains many of the outstanding names in analytical chemistry. This book is a must for the appropriate libraries and should be placed in such a position that chemists, and analytical chemists in particular, are tempted to browse among its pages.

C. S. G. Phillips

C. S. G. Phillips is Lecturer in Inorganic Chemistry at the University of Oxford, UK.

● *Science, Technology and Society: A Cross-Disciplinary Perspective* (reviewed in the 10 November issue of *Nature*: 270, 126; 1977) is published for the ICSPS by Sage Publications: London and Beverly Hills, California.

● *Theory of Computer Science* (reviewed in the 3 November issue of *Nature*: 270, 85; 1977) is published by Chapman and Hall: London and distributed in the USA by Halsted: New York.

Problems of desertification

Desertification: Environmental Degradation in and around Arid Lands. Edited by Michael H. Glantz. Pp. xiv+346. (Westview: Boulder, Colorado, 1977.) \$20.

THE United Nations Water Conference, held in Argentina in March, 1977, and the United Nations Conference to Combat Desertification, held in Kenya in August, 1977, reflect the worldwide attention now being paid to the destruction of arable or potentially arable land throughout the arid and semi-arid regions of the world. The volume under review comprises a collection of articles by specialists in a range of disciplines which examine and evaluate the social, political, economic, environmental and technical problems related to the causes and effects of desertification. Most of them are concerned, one way or another, with the Sahara and the Sahel savanna of Africa.

In the first of these articles, the editor outlines the approach of the United Nations to "desertification" or, as H. N. Le Houérou prefers to call it, "desertization". Glantz defines this as a global "environmental problem which is primarily national in cause and national in effect". The Sahelian states are among the poorest in the world and, therefore, "in need of financial, technical, moral and other support if they are to have any chance whatsoever to cope effectively with the problems that are linked to desertification within their borders". No-one, however, explains how one should refute the logic of the Sahelian peoples—if, during the last drought, two thirds of their cows died, then, by raising three times as many cattle, the original number might be expected to survive the next drought!

At least half of all the timber cut in the world is used as fuel for cooking or as a source of warmth. Eric P. Eckholm discusses the problem posed by an Indian official: "even if we somehow grow enough food for our people in the year 2,000, how in the world will they cook it?" The suicidal deforestation of Asia, Africa and Latin America will have to be reversed. The problem of ecological deterioration in Niger is discussed by James T. Thomson; that of pastoral development in Somalia by Jeremy Swift. Authors of other chapters include Richard W. Katz, William W. Kellogg, Stephen H. Schneider and Helen Ware. Surprisingly, the Interim Report of the South African Drought Investigation Commission of April, 1922, is reprinted,

without comment, as chapter 10. This historical document shows that neither today's problems, nor their solutions, are new—although the recommendation that jackals should be exterminated might no longer win universal approval.

The review of problems of desert land reclamation in the USSR, by A. G. Babayev, includes an appendix consisting of abstracts of 24 papers published after a symposium held at Ashkhabad, in 1976. Babayev concludes that scientists "must now concentrate on determining the optimum limits of harmonic development of society and nature". "The Communist Party and the Soviet government" seem to have learned that nature may be tamed, but cannot be conquered by man!

Transport properties of simple liquids

Classical Kinetic Theory of Fluids. By P. Résibois and M. De Leener. Pp. xv+412. (Wiley: New York, London and Toronto, 1977.) £22.

"AMONG the many available texts on statistical mechanics, few, if any, give the reader a coherent and sustained introduction to the various methods that have made non-equilibrium statistical mechanics so successful." This quotation from the preface understates the difficulties facing a research worker in the field. If he tries to piece together the original literature, he meets mysterious concepts like "plateau time", "friction constant" and "auto-correlation functions"; and he can have a hard time in deciding whether a theory contains, or does not contain, adjustable constants.

This book contains a connected account of the various approaches to the problem of predicting the transport coefficients of a "simple" liquid. Nearly all the work is concerned with the rigid sphere and n 'th power repulsion models. Theoretical predictions are compared with "computer experiments" rather than with measurements on the rare gases. This is because of the complicated actual forces between real molecules of "simple" liquids, and also because information such as the time variation of correlation functions can be obtained directly from computer experiments, but can only be inferred indirectly from neutron scattering experiments on real liquids.

The only proper way of dealing with the famous "irreversibility paradox" is to proceed via Liouville's equation. This is done in section C of the book,

A book on deserts seems an unlikely place in which to find a discussion of the world's oceans, but J. Dana Thompson's interesting chapter examines the notion of biological deserts in the ocean, and assesses the limitations of the sea as a biological resource which, however, could be made somewhat more productive by mariculture, the reclamation of polluted areas, harvesting unconventional species, and the abolition of over-fishing. As in the case of all the problems of environmental degradation, we know *what* should be done but not *how* it could be accomplished. The matter is one of individual responsibilities.

J. L. Cloudsley-Thompson

J. L. Cloudsley-Thompson is Professor of Zoology at Birkbeck College, University of London, UK.

after two introductory sections on stochastic processes and on various forms of the Boltzmann equation. The Boltzmann equation has essentially irreversible solutions, and the central problem is to show that such an equation can be consistently derived from Liouville's equation by proceeding to the thermodynamic limit in a large assembly.

This programme cannot be carried through rigorously for any physically realistic model, but section C makes it clear that most, if not all, of the difficulties of principle have been gradually overcome, and that the remaining difficulties are almost certainly technical. The reader is led through some far from simple work with great clarity, and the style is pleasant and informative. No attempt is made to disguise the difficulties still outstanding—for example, the long term behaviour of the correlation functions and the fact that some treatments give transport coefficients that vary non-analytically with density (or diverge). Paradoxically, such difficulties seem to be particularly severe for two-dimensional assemblies. A final section discusses the, more formal, theory of transport coefficients based on time-dependent correlation functions.

The appearance of the book is most timely. It is splendidly printed and produced and there is a good index and some clear figures. The bibliography is surprisingly short but probably adequate, consisting largely of books and review articles rather than original sources. The authors have certainly achieved the objectives set out in their preface.

H. N. V. Temperley

H. N. V. Temperley is Professor of Applied Mathematics at University College, Swansea, UK.